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Development and utilization of molecular markers to study genetic diversity of smooth cordgrass and cold tolerance sugarcane

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DEVELOPMENT AND UTILIZATION OF MOLECULAR MARKERS TO STUDY GENETIC DIVERSITY OF SMOOTH CORDGRASS AND COLD TOLERANCE IN SUGARCANE

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"…Live your life each day as you would climb a mountain. An occasional glance toward the summit keeps the goal in mind, but many beautiful scenes are to be observed from each new vantage point. Climb slowly, steadily, enjoying each passing moment; and the view from the summit will serve as a fitting climax for the journey…"

(Harold V. Melchert)

To my dear and loved parents, Flor and David, for their constant and endless love, for trusting my choices, and to whom I owe all that I am and I dedicate this work.

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Abbreviations

AMOVA	analysis of molecular variance
APS	ammoniumperoxidisulfate
bp	base pair
DEG	differentially expressed gene
dNTP	deoxynucleotidetriphosphate
EST	expressed sequence tag
eSSR	EST-derived simple sequence repeat
gSSR	genomic simple sequence repeat
IPTG	isopropyl β-D-1-thiogalactopyranoside
MAS	marker-assisted selection
PCR	polymerase chain reaction
RAPD	random amplified polymorphism DNA
RT	reverse transcription
Sq/q	semiquantitative/quantitative
SSR	simple sequence repeat
Ta	annealing temperature
TAE	tris acetate EDTA
TBE	tris borate EDTA
TEMED	tetramethyl-ethylenediamine

Abstract

Smooth cordgrass (*Spartina alterniflora* Loisel) and sugarcane (*Saccharum* spp. hybrids) are both grasses that belong to the family Poaceae and have significant impact on Louisiana environment and economy. Coastal Louisiana accounts to ~80% of the national wetland loss through natural and human interventions; (re)vegetation is considered an important component of coastal restoration efforts. Smooth cordgrass, a warm season marsh grass, is used as an ecosystem engineer to stabilize and restore Louisiana coastal marshes. In this study, genetic diversity was determined among 13 elite smooth cordgrass accessions of Louisiana through 276 polymorphic markers generated by 23 SSR and 32 RAPD primers. AMOVA results showed that sufficient variation existed among smooth cordgrass accessions for further exploitation in breeding program. A few genetic markers unique to specific smooth cordgrass accession(s) were also developed. Sugarcane, the number one row crop in Louisiana, is sensitive to cold; so cold tolerance is desirable to expand its cultivation in more northern environments. Cold tolerance alleles of *Saccharum spontaneum* can be exploited to develop sugarcane clones tolerant to low temperatures. To this end, forty differentially expressed genes (DEGs; 29 up-regulated and 11 down-regulated) were identified in a cold-tolerant clone Ho02-144 and a cold-sensitive clone L79-1002 under cold stress, using annealing control primer system. Reverse-transcription PCR (RT-PCR) analysis of the cold-responsive DEGs revealed distinctive expression profiles in the tolerant and sensitive clone. Mining of the cold-responsive DEGs yielded 12 sequences with simple sequence repeats (SSRs), which were used to design eSSR primers. Genotyping of 48 Louisiana sugarcane clones and 16 ancestral parents with these eSSRs generated 170 polymorphic markers that could classify cultivated sugarcane clones from their ancestral *S. spontaneum* clones at 0.23 similarity coefficient. The cold responsive genes will be useful for breeding cold tolerant sugarcane either through genetic engineering or selection through the use of gene-based markers.

Chapter I
General Introduction

1.1 Background of the Study

Coastal wetlands of Louisiana are considered a national treasure for various reasons: they protect multi-billion dollar oil and gas industries; provide nursery grounds for fish and winter habitat for about one-half of the Mississippi Flyway waterfowl population; and more importantly serve as a buffer from hurricanes and storms (CWPPRA, 2012). Forty percent of the nation's coastal marshes are in Louisiana and at the same time 80% of the total wetland loss occurs in Louisiana due to human and natural interventions (CWPPRA, 2012). Wetland restoration projects are being implemented as the best alternative to improve water quality and alleviate degradation of natural wetlands (Mitsch and Gosselink, 2000). Many of these restoration projects require the utilization of native plants such as smooth cordgrass because it is the most dominant intertidal saline marsh plant.

Smooth cordgrass, also called salt marshgrass, is a warm-season grass member of family Poaceae that grows along the Atlantic and Gulf coasts of North America (Valiela et al., 1978). This species is important as an ecosystem engineer in Louisiana for stabilization and rescue of coastal marshes. This is due to its aggressive spreading ability through rhizomes to form a dense canopy, and tolerance to extreme salinity and other environmental stresses (Daehler and Strong, 1997; Walkup, 2011). Smooth cordgrass grows out into the water at the seaward edge of a salt marsh, and accumulates sediment and enables other habitat-engineering species, such as mussels, to settle (Valiela et al., 1978). However, until recently, only two smooth cordgrass cultivars (Vermilion and Bayshore) have been released commercially. "Vermilion" has demonstrated superior growth characteristics and has performed well in highly disturbed salt marshes, but its extensive use as a monoculture in the coastal habitat restoration activities in Louisiana (Utomo et al., 2009) is a concern in terms of its genetic vulnerability to biotic and abiotic stresses. Therefore, there is a need for developing new genetically diverse smooth cordgrass cultivars for wetlands restoration

capable of adapting to different environmental changes (Baisakh et al., 2009). Currently, the coastal plants research program of the Louisiana State University Agricultural Center (LSU AgCenter) is evaluating a wide collection of native smooth cordgrass accessions throughout Louisiana (Fang et al., 2004) over a range of coastal environments for germination, seedling survival, seed production, and other traits of significance such as salt stress tolerance, for their use in marsh restoration.

Sugarcane, another member of the Poaceae family, is the leading row crop in Louisiana agriculture; it produces about 19 percent of total sugar production grown in the United States, and provides employment to approximately 32,000 people (Salassi et al., 2010). The sucrose, extracted and purified in factories, is processed into sugar for use in human food and food industries or is fermented to produce ethanol, as in Brazil, which makes it a first generation feedstock for the biofuel industry (Vettore et al., 2003). Recently, it has gained special attention as a second generation energy crop due to its ability to produce high-biomass (Pinto et al., 2010).

Cold stress is one of the serious abiotic factors that can cause adverse effects on the growth, productivity and geographical distribution of crops such as sugarcane (Menossi et al., 2008). Sugarcane is grown in warm environments surrounded by water and is sensitive to cold, but the existence of genetic variability in response to cold stress among the commercial sugarcane varieties, especially those with cold-tolerant alleles from *Saccharum spontaneum*, can be exploited to produce low temperature-tolerant sugarcane plants (Du et al. 1999). Integration of cold tolerance in the sugarcane crop will allow expansion of its cultivation to colder environments. Therefore, cold tolerance is considered as a valuable trait in Louisiana sugarcane breeding program to develop improved and superior varieties.

Identification of genes responding to a particular environment such as cold temperature in sugarcane will contribute to the basic understanding of the underlying genetic and physiological mechanisms of cold tolerance. Also, these genes can be used for development of allele-specific DNA markers for application in conventional breeding or in genetic engineering for development of cold tolerant sugarcane (Manners and Casu, 2011).

1.2 Genetic Diversity and Genetic Markers

Genetic diversity is the variation among genotypes within a population or among populations, which is reflected by morphological, physiological and genetic differences due to differences in allelic compositions (Frankham et al., 2002). Understanding the genetic diversity or germplasm organization of important plant species, such as smooth cordgrass and sugarcane, is important for more efficient selection of improved cultivars and to maintain genetic gain, which is essential to the success of a breeding program (Swanson, 1996). More emphasis is being given to increasing the genetic base of the smooth cordgrass and sugarcane in Louisiana breeding programs because there is concern of inbreeding depression and vulnerability to environmental catastrophe in the way modern cultivars are bred. The genetic diversity studies of these species will help breeders select diverse parents to generate viable superior crops (Hamrick, 2004).

Genetic diversity studies have been made easier with the advent of DNA-based molecular markers that demonstrated better selection efficiency for plant improvement. DNA marker profiles provide reliable and direct strategies for assessing the genetic variation within populations for population structure and genetic identification (Morgante and Olivieri, 1993). The development of unique genetic markers to identify individual varieties (genotype identification) and DNA fingerprinting will help to preserve the integrity and purity of improved and released varieties.

Depending on the objectives, several different types of molecular marker systems have been used for genetic diversity, population structure analysis, genotype identification development as well as mapping and marker-assisted selection. Random amplified polymorphic DNA (RAPD) markers are easy and could be used for genetic diversity studies and subsequent genotype ID development through sequence characterized amplified region (SCAR) markers (Ryan, 2003). On the other hand, microsatellites or simple sequence repeats (SSRs) derived from both coding (eSSR) and non-coding (gSSR) regions of the genome are highly informative, reproducible markers. These have been successfully used in genetic mapping and development of markers for marker-assisted selection of desirable traits in crops (Gupta and Varshney, 2000). Another advantage of these genic eSSRs is their transferability across species that

makes them useful in comparative genomics (Varshney et al., 2005). Therefore, the development of molecular markers will be useful for the research community engaged in wetlands plants and sugarcane programs, especially when these can be targeted at genomic regions known to contribute to the control of economically important, genetically complex traits such as abiotic stress (such as cold and salt) tolerance (Baisakh et al., 2009). Also, the characterization of the gene expression profiles under stress is an important tool for the development of genic markers for their use in breeding cold-tolerant sugarcane.

Given the importance of smooth cordgrass and sugarcane in Louisiana as described above, the present investigation has been focused on these two grass species. Chapter II describes the genetic diversity studies and development of unique genetic tags of the elite smooth cordgrass accessions including three very recently registered lines of smooth cordgrass (Knott et al., 2012). Chapter III encompasses gene expression studies in sugarcane for identification of cold-responsive genes and subsequent development of genic markers for characterization of the Louisiana sugarcane germplasm.

Chapter II
Genetic Diversity and Identification of Genotype Specific Alleles of *Spartina alterniflora* Loisel Collections of Louisiana Using RAPD and SSR Markers

2.1 Introduction

Coastal Louisiana accounts for approximately 40% of the nation's coastal wetlands in the lower 48 states. Wetlands are areas where water covers the soil or is near the surface of the soil (Fang, 2002). They improve the water quality by filtering out pollutants and absorbing nutrients; act as storm buffer against hurricanes and storms, as flood control devices by holding excess floodwaters during high rainfall, and as habitat for fish and wildlife (CWPPRA, 2012). These wetlands are in jeopardy because Louisiana loses about 3.5 million acres of wetlands, or about 75 to 91 square kilometers per year, which is nearly 80% of the total coastal wetland loss (CWPPRA, 2012). If coastal wetland loss continues at this rate, by the year 2050 Louisiana would lose an additional 2132 square kilometers of coast marshes, which will deprive to Louisiana of fish, wildlife, and other environmental benefits (CWPPRA, 2012).

Smooth cordgrass (*Spartina alterniflora* Loisel) is a warm-season perennial grass that belongs to the genus *Spartina* in the Poaceae family. Smooth cordgrass is found along the Atlantic and Gulf coast of North America where it forms dense colonies, especially in estuarine salt marshes (Valiela et al., 1978). The importance of this species in Louisiana has been primarily for use in coastal erosion control and protection of shorelines, canal banks, levees, and other areas of soil-water interface. These salt marshes are meant to protect wetlands lost by the natural and/or human interventions due to its vigorous spreading ability and strong underground rhizome growth (Duncan and Duncan, 1987). The importance of smooth cordgrass as an environmental engineer is based on its rapid growth, high capacity to tolerate various stresses, its great adaptation to many soil types filtering heavy metals and toxic materials from the water column (Walkup, 2011).

Coastal restoration projects in Louisiana rely heavily on the utilization and reintroduction of native plants such as smooth cordgrass in order to offset the wetland loss because it is the predominant marsh plant that determines the estuarine structure and ecological function in these regions (Proffitt et al., 2003; Utomo et al., 2009; Knott et al., 2012). Recently there is an increased interest of using smooth cordgrass in restoration projects in Louisiana, which will increase in the following years because the Coastal Wetlands Planning, Protection and Restoration Act (CWPPRA) has released a project for vegetation of smooth cordgrass across the entire Louisiana coast (CWPPRA, 2012).

So far, two smooth cordgrass cultivars have been released by the Unites States Department of Agriculture's National Resources Conservation Service (USDA-NRCS) for coastal restoration programs: Bayshore (Hamer et al., 1994) was released for the Atlantic coast and Vermilion (CP8; Fine and Thomassie, 2000) was released for the Gulf of Mexico coast. Vermilion has demonstrated superior growth characteristics and has performed well in highly disturbed salt marshes. It is the only cultivar that has been grown extensively for coastal restoration in Louisiana (USDA NRCS, 2006; Utomo et al., 2009), which is a concern because the use of a single cultivar could have serious repercussions in terms of genetic vulnerability to biotic and abiotic stresses. Therefore, there is an increased demand for developing new genetically diverse smooth cordgrass cultivars with ability to adapt to changing environment for wetlands restoration and reclamation (Baisakh et al., 2009). The genetic diversity studies of Louisiana smooth cordgrass will allow the researchers to develop strategies to generate viable marshland systems and increase ecosystem functionality through development of improved smooth cordgrass varieties (Utomo et al., 2009; Umamaheswari et al., 2010).

The knowledge on the understanding of the genetic diversity in germplasm organization is important for more efficient selection of improved cultivars. Gene expression studies in smooth cordgrass have resulted in identification of key genes involved in its salt and heat stress responses (Baisakh et al., 2006; 2008; 2009). Being extremely salt tolerant and a member of grass family that constitutes the majority of the food crops, this grass has been proposed as a model halophyte for bioprospecting genes to improve salt tolerance in cereal crops (Subudhi and Baisakh, 2011).

Louisiana State University Agricultural Center (LSU AgCenter) coastal plant breeding program is the first and only program established in the state of Louisiana and the United States, which is aimed at improving Louisiana native plants, and developing certification standards of native plant varieties, modeled after agricultural systems. Currently, the LSU AgCenter coastal plants breeding program is evaluating a wide collection of smooth cordgrass accessions from South Louisiana (Fang, 2002) over a range of coastal environments for germination, seedling survival, seed production, and other traits of significance such as salt stress tolerance, for their use in restoration activities. Thirteen of these accessions were germinated and selected under field conditions for different agronomic traits (Ryan et al., 2007). Three elite derivatives of these accessions, maintained as pure foundation material, were registered recently as clonal varieties for use in marsh restoration (Knott et al., 2012) and three more are in the registration pipeline.

DNA-based markers are extremely useful for a variety of purposes and their efficacy is determined by the technology that is used to reveal DNA polymorphisms based on the variation of nucleotide sequences (Mazur and Tingey, 1995). Neutral markers such as amplified fragment length polymorphism (AFLP; Vos et al., 1995), simple sequence repeats (SSR; Parida et al., 2006), expressed sequence tags (EST)-derived SSR (eSSR; Baisakh et al., 2009), random amplification polymorphic DNA (RAPD; Stiller and Denton, 1995), target region amplification polymorphism (TRAP; Hu and Vick, 2003) and sequence-related amplification polymorphism (SRAP; Li and Quiros, 2001) have been routinely used to screen populations for diversity and mapping studies.

Random amplification polymorphic DNA (RAPD) markers are simple, quick, easy to assay, and more importantly do not require sequence information of the target for design of the primers. They are generally dominant markers with high genomic abundance because of their random distribution throughout the genome (Williams et al., 1990). In addition, RAPD assay requires low quantities of DNA for amplification by polymerase chain reaction (PCR) and may facilitate more effective diversity analysis (Karp and Edwards, 1997). On the other hand, microsatellites or simple sequence repeats (SSRs) are short sequences (2-6 base motifs) of repetitive DNA found in the coding and non-coding regions of the genome (Parida et al., 2006). The genomic SSR

markers (gSSR) are highly variable within a species and are often used for studying polymorphism in different plant species. At the same time cDNA clones or expressed sequence tags (EST) deposited in public databases such as GenBank, can be searched for identification of SSRs, which are known as EST-derived SSRs (eSSR). The eSSR markers allow tracking of the genetic loci controlling the useful traits without extensive field evaluation because of the fact that they reside directly within the gene(s) controlling the trait(s) of interest and they can be used as diagnostic markers for the genes or to map them (Chen et al., 2006; Wen et al., 2010). SSRs are co-dominant molecular markers that are being increasingly used due to their robustness, reproducibility, uniform distribution, polymorphism, ease of use, and relatively simple interpretation (Cholastova et al., 2011).

RAPD and SSR markers have been used to assess the genetic variability of smooth cordgrass. Using RAPDs, O'Brien et al. (1999) observed that smooth cordgrass could be classified into three geographic areas (New England, South Atlantic, and Gulf coast). RAPD markers were used to test the hybrid status between the native and introduced *Spartina* species in the greenhouse and in the field (Daehler and Strong 1997). The authors identified introgression and spread of hybrids as a risk to conservation of *S. foliosa* in San Francisco Bay. Daehler et al. (1999), using RAPDs, reported the spread of a dwarf ecotype of smooth cordgrass in San Francisco Bay. They found that the ecological range of this ecotype is similar to that of *S. patens*, suggesting that its absence in San Francisco Bay has allowed the dwarf ecotype of smooth cordgrass to survive and spread. Ryan et al. (2007) evaluated the genetic diversity of accessions collected from South Louisiana smooth cordgrass populations using RAPD and AFLP markers. Cluster analysis showed natural variation among the collections contributing to plant establishment from seed. Travis et al. (2002) characterized genetic diversity of smooth cordgrass from restored wetland and undisturbed wetland sites, and established that genetic diversity in restored populations is comparable to natural populations. Likewise, the development and use of SSR markers has revealed important findings in smooth cordgrass. Blum et al. (2004) and Sloop et al. (2005) developed genomic SSR markers to ascertain the ecological and evolutionary origin among native and invasive species of smooth cordgrass. Using SSRs, Blum et al. (2007) suggested existence of genetic differences among smooth cordgrass populations from mid- and

southern Atlantic regions. Baisakh et al. (2009) proposed that EST-derived SSRs (eSSRs) of smooth cordgrass were transferable across other species of *Spartina* and could facilitate comparative genomic studies of *Spartina* species with different origin and environmental adaptation. The eSSRs in combination with gSSRs have been used for genetic diversity analysis, genome mapping, and evolutionary studies in *Spartina* (Blum et al., 2004; Blum et al., 2007; Sloop et al., 2005; Baisakh et al. 2009).

Molecular investigations into the population structure, gene diversity and genetic relatedness within and among smooth cordgrass accessions will be of immense value to identify genetically diverse accessions as well as cataloging genetic fingerprints of genotypes for maintaining and monitoring genetic integrity and purity of future released accessions (Baisakh et al., 2009). The importance and use of genetic markers unique to these lines for DNA fingerprinting will help preserve the genotype identification (ID) of the released material. The knowledge of the ID of smooth cordgrass plants is an important component of the seed certification to ensure identify and purity of plant materials, which is essential in the development of a new foundation plant production industry in Louisiana.

2.1.1 Research Objectives

With a long-term goal of developing genetic markers to facilitate breeding of improved smooth cordgrass varieties for their use in coastal wetlands restoration, the present study was undertaken with the following objectives:

- To assess genetic diversity among and within a set of 13 smooth cordgrass elite accessions using RAPD and SSR markers;
- To identify a unique genetic tag for a particular clone/accession.

2.2 Materials and Methods

2.2.1 Plant Material

Thirteen smooth cordgrass CP (crossing plot) accessions (CP1, CP2, CP3, CP4, CP5, CP6, CP7, CP8, CP9, CP10, CP11, CP12 and CP13) including six soon-to-be-released varieties (Table 2.1) were used in this study. One of these accessions, Vermilion (CP8; Fine and Thomassie, 2000) is used extensively in Louisiana coastal reclamation and restoration projects along the Gulf coast. Seven accessions (CP1-CP7) were selected from 126 accessions that were collected as seeds from different regions of South Louisiana (Fang et al., 2004) in 1998, and grown in 1999. Based on the results of germination, seedling survival, and seedling vigor tests, 20 accessions were selected for preliminary field evaluation. In 2000, 20 plants per accession (400 plants) were field evaluated at the LSU AgCenter's Central Research Stations, Ben Hur, Baton Rouge, LA (Ryan et al., 2007). In 2001, forty lines were selected based on their plant height, spread, disease reaction, and plant vigor data in advanced field trial (Ryan et al., 2007). The other five accessions (CP9-CP13) were developed in a crossing block through open-pollination in 1999. All thirteen accessions were vegetatively maintained in a greenhouse of the LSU AgCenter, Baton Rouge, LA. Five individual plants from each accession were also vegetatively maintained inside the greenhouse for analysis with SSR markers. The accessions show contrasting phenotypes with respect to different agronomic traits including seedling vigor and spread, seed set etc (Knott et al., 2012).

2.2.2 DNA Isolation

Total genomic DNA was extracted from fresh, young leaves of five plants from each of 13 smooth cordgrass accessions using the small scale CTAB protocol (Doyle and Doyle, 1990). DNA quality and quantity was determined by agarose (1 % w/v) gel electrophoresis and a ND-100 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), respectively. Genomic DNA was resuspended at a 50 ng/µl working concentration for use in RAPD and SSR analysis. The concentrated DNA stock and the working DNA solution was stored at -20 ^{o}C and 4 ^{o}C, respectively.

Table 2.1. List of the 13 smooth cordgrass accessions used in SSR and RAPD analysis

Alias	Material	Accession No.	Origin	Source
CP1	seed	98NR7-00BH-12	Lafourche	LSU AgCenter Coastal Plants Program
CP2	seed	98NR8-00BH-5	Lafourche	LSU AgCenter Coastal Plants Program
CP3	seed	98NR26-00BH-18	Lafourche	LSU AgCenter Coastal Plants Program
CP4	seed	98NR82-00BH-19	Cameron	LSU AgCenter Coastal Plants Program
CP5	seed	98NR47-00BH-16	Cameron	LSU AgCenter Coastal Plants Program
CP6	seed	98NR27-00BH-13	Terrebonne	LSU AgCenter Coastal Plants Program
CP7	seed	98NR99-00BH-3	Jefferson	LSU AgCenter Coastal Plants Program
CP8	seed	Vermillion clone	Vermillion	LSU AgCenter Coastal Plants Program
CP9	seed	99RS08	Polycross of Line 1-8	LSU AgCenter Coastal Plants Program
CP10	seed	99RS12	Polycross of Line 1-8	LSU AgCenter Coastal Plants Program
CP11	seed	99RS14	Polycross of Line 1-8	LSU AgCenter Coastal Plants Program
CP12	seed	99RS22	Polycross of Line 1-8	LSU AgCenter Coastal Plants Program
CP13	seed	GT76	Polycross of Line 1-8	LSU AgCenter Coastal Plants Program

2.2.3 Genetic Fingerprinting

2.2.3.1 RAPD Analysis

One hundred ng of genomic DNA was used as a template for polymerase chain reaction (PCR) of the 13 smooth cordgrass accessions by forty-nine random primers (Table 2.2) (Operon Technologies, Alameda, CA). All PCR reactions were conducted in a final volume of 25 µl, containing 1X PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer, 1.0 U *Taq* DNA polymerase (Promega, Madison, WI) and ddH_2O. Amplifications were performed in a Thermocycler (BIORAD, Hercules, CA) using a profile: (1) initial denaturation 94 °C, 3 min; (2) 45 cycles of 94 °C, 1 min; 36 °C, 1 min; 72 °C, 2 min; and (4) final extension 72 °C, 5 min. The amplified products were resolved on a 1.5 % (w/v) agarose gel in 1X- TAE buffer following ethidium bromide staining, and photographed using the KODAK Gel Logic 200 Imaging system (Kodak, New Haven, CT).

2.2.3.2 SSR Analysis

Sixty one SSR primers (44 gSSR and 17 eSSR) were used to fingerprint the smooth cordgrass accessions and five individual plants from each accession. The gSSR markers were developed by Blum et al. (2004) and Sloop et al. (2005), and the eSSR markers were developed by Baisakh et al. (2009). All PCR amplifications were carried out in a 25 µl final reaction volume, containing 20 ng genomic DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer, 1.0 U *Taq* DNA polymerase (Promega, Madison, WI), and ddH_2O. Amplifications were performed in a thermal cycler (BIORAD, Hercules, CA) with the following amplification profile: (1) initial denaturation 95 °C, 4 min; (2) 35 cycles of 94 °C, 45 s; Ta °C, 45 s; 72 °C, 1 min; and (4) final extension 72 °C, 5 min. The amplified products were separated in a 4.5 % (w/v) SFR agarose (Amresco, Solon, OH) gel in 1X- TAE buffer for 4.5 h at 110 V or 15 h at 45 V, and stained with ethidium bromide. Images were analyzed under UV light using the KODAK Gel Logic 200 Imaging system (Kodak, New Haven, CT).

Table 2.2. List of RAPD primers used for genotyping of the 13 smooth cordgrass accessions

Operon Primer	Sequence 5'-3'	Operon Primer	Sequence 5'-3'
OPA1	CAGGCCCTTC	OPB12	CCTTGACGCA
OPA2	TGCCGAGCTG	OPB15	GGAGGGTGTT
OPA3	AGTCAGCCAC	OPB20	GGACCCTTAC
OPA4	AATCGGGCTG	OPC20	ACTTCGCCAC
OPA5	AGGGGTCTTG	OPD5	TGAGCGGACA
OPA6	GGTCCCTGAC	OPD13	GGGGTGACGA
OPA7	GAAACGGGTG	OPD16	AGGGCGTAAG
OPA8	GTGACGTAGG	OPE9	CTTCACCCGA
OPA9	GGGTAACGCC	OPF8	GGGATATCGG
OPA10	GTGATCGCAG	OPF14	TGCTGCAGGT
OPA11	CAATCGCCGT	OPG2	GGCACTGAGG
OPA13	CAGCACCCAC	OPG12	CAGCTCACGA
OPA14	TCTGTGCTGG	OPH12	ACGCGCATGT
OPA15	TTCCGAACCC	OPJ1	CCCGGCATAA
OPA16	AGCCAGCGAA	OPJ6	TCGTTCCGCA
OPA17	GACCGCTTGT	OPT17	CCAACGTCGT
OPA18	AGGTGACCGT	OPW19	CAAAGCGCTC
OPA19	CAAACGTCGG	OPY17	GACGTGGTGA
OPA20	GTTGCGATCC	OPX3	TGGCGCAGTG
OPB3	CATCCCCCTG	OPX4	CCGCTACCGA
OPB4	GGACTGGAGT	OPX5	CCTTTCCCTC
OPB6	TGCTCTGCCC	OPX10	CCCTAGACTG
OPB8	GTCCACACGG	OPX11	GGAGCCTCAG
OPB10	CTGCTGGGAC	OPX15	CAGACAAGCC
OPB11	GTAGACCCGT		

2.2.4 Data Analysis

All visibly detectable polymorphic and monomorphic bands in the 13 accessions of smooth cordgrass were scored for the presence (1) or absence (0) from the RAPD and SSR profiles, and the data were converted into a binary matrix. The degree of polymorphism for each RAPD and SSR marker was determined by measuring their polymorphism information content (PIC), which was estimated using the formula (Botstein et al., 1980): PIC = 2 fi (1- fi); where fi is the frequency of the amplified alleles (bands present), and (1- fi) is the frequency of the null alleles (including no amplification).

Pair-wise genetic similarity among individual accessions was analyzed for both RAPD (GS_{RAPD}) and SSR (GS_{SSR}) markers using the Jaccard's similarity coefficient of the SIMQUAL module (Jaccard, 1908) of the Numerical Taxonomy System (NTSYSpc) Version 2.2 software (Rohlf, 2005). The resultant matrix was employed for clustering analysis to create a dendrogram (tree) based on the UPGMA (unweighted pair group method with arithmetic means) with the SAHN module. Correlation between the two matrices obtained with the RAPD and SSR marker types was estimated as a cophenetic correlation coefficient (r) using the Mantel's test of matrices comparison with 1000 permutations (Mantel, 1967). The MxComp module was used to compare the original similarity matrix that was clustered with the cophenetic value matrix. Confidence for each dendrogram was supported by a bootstrap analysis with 1000 iterations using WinBoot software (Yap and Nelson, 1996).

Principal coordinate analysis (PCoA) was also performed to estimate the genetic distance between each group of the accessions, based on the estimated similarities, using the DCENTER, EIGEN and PROJ modules of the NTSYSpc program. To assess variations among and within accessions of smooth cordgrass and the fixation index (FST), an analysis of molecular variance (AMOVA) was performed using Arlequin v.3.5 (Excoffier et al., 2010), which allows the partition of the total variation into within- and among- group variation components.

2.3 Results

Analysis of genetic diversity/relatedness among 13 smooth cordgrass accessions was performed for RAPD and SSR marker systems separately or in combination (Table 2.3).

Table 2.3. Polymorphism information and comparison of RAPD and SSR markers in the 13 smooth cordgrass accessions

Index with abbreviations		Marker system		
		RAPD	SSR	Total
Number of primer used	U	49	61	110
Number of primers w/o amplification		10	11	-
Number of monomorphic primers	Pnp	7	27	-
Number of polymorphic primers	Pp	32	23	55
Number of polymorphic bands	Np	208	68	276
Number of monomorphic bands	Nnp	155	52	207
Average number of bands/primer	Np+Nnp/Pp+Pnp	9.3	2.4	-
Average number of polymorphic bands/primer	Np/Pp	6.5	2.96	-
Percent of polymorphism	%	57.3	56.7	100
Number of loci	L	32	23	55
Number of loci/assay	Na	0.65	0.38	-
Polymorphic information content	PIC	0.35	0.33	-

2.3.1 Level of Polymorphism by RAPD and SSR Markers

A total of 483 loci were amplified by 49 RAPD and 61 SSR primers, of which 276 (57.14%) were polymorphic. The number of loci per primer ranged from 5 to 25 for RAPD (Table 2.4) and 2 to 9 for SSR (Table 2.5). The polymorphism of the 13 smooth cordgrass accessions detected by RAPD markers was slightly higher than that detected by SSR markers. Ten of the arbitrary RAPD primers did not result in reproducible amplification, while the remaining 39 primers generated 363 fragments, with an average of 9.3 fragments per primer (Table 2.3). Thirty two (65.3 %) out of the 39 primers were polymorphic among the accessions that generated 208 (57.3 %) polymorphic loci (Table 2.3). Of the 61 SSR primers, eleven failed to amplify, while the remaining 50 primers detected a total of 120 loci, with an average of 2.4 loci per primer (Table 2.3). Of these 120 bands, 68 (56.7 %) loci generated by 23 (37.7%) primer pairs were polymorphic among the accessions (Table 2.3).

The number of polymorphic fragments per primer varied of 1 to 18 for RAPD with an average of 6.5 polymorphic bands per primer, while for SSR markers the number of loci for each primer varied from 1 to 6, with an average of 2.96 loci per marker (Table 2.3). Most of the SSR loci were bi-allelic in nature. The polymorphic information content (PIC) values ranged from 0.14 to 0.50 with a mean of 0.35 for the RAPD markers and from 0.14 to 0.50 with an average of 0.33 for the SSRs markers (Table 2.3). The highest number (18) of loci was amplified by the RAPD primer OPA1. The primers OPA1 and OPF14 amplified the maximum percent (78 and 80%, respectively; Table 2.4) of polymorphic loci, while for SSR markers six loci were detected with only one SSR primer (Spar41; Table 2.5). The DNA patterns produced by RAPD and eSSR markers are shown in Figure 2.1.

2.3.2 Identification of Accession Specific Allele (Genotype ID/Unique Tags)

Out of thirty-two polymorphic RAPD primers, 17 amplified loci that were specific to 10 CP accessions (CP1, CP3, CP4, CP5, CP7, CP8, CP9, CP10, CP12 and CP13) (Table 2.6). Two accessions (CP7 and CP9) were distinguished from the rest of

Table 2.4. Number of total fragments and polymorphism rate generated by the RAPD primers in the 13 smooth cordgrass accessions

RAPD Primer	Total Fragments	Polymorphic Fragments	Monomorphic Fragments	Polymorphism Rate (%)
OPA1	23	18	5	78
OPA2	13	7	6	54
OPA3	21	13	8	62
OPA4	25	14	11	56
OPA5	10	6	4	60
OPA7	11	6	5	55
OPA8	8	4	4	50
OPA13	9	4	5	44
OPA16	11	6	5	55
OPA17	8	4	4	50
OPA18	12	9	3	75
OPA19	13	9	4	69
OPA20	12	7	5	58
OPB3	12	7	5	58
OPB4	12	8	4	67
OPB6	11	6	5	55
OPB8	12	6	6	50
OPB10	9	4	5	44
OPB11	11	6	5	55
OPB12	12	8	4	67
OPB15	7	4	3	57
OPC20	10	6	4	60
OPF14	5	4	1	80
OPG2	7	1	6	14
OPH12	9	5	4	56
OPJ1	7	2	5	29
OPJ6	10	7	3	70
OPT17	10	5	5	50
OPX3	9	4	5	44
OPX4	11	7	4	64
OPX15	12	7	5	58
OPW19	11	4	7	36
Total	363	208	155	-
Mean	11	6.5	5	57.30

Table 2.5. Number of total fragments and polymorphism rate generated by the SSR primers in the 13 smooth cordgrass accessions

Polymorphic markers	Total Fragments	Polymorphic Fragments	Polymorphism (%)
eSSR21	5	2	40
eSSR29	6	2	33
eSSR35	6	3	50
eSSR58	5	2	40
eSSR64	2	1	50
SPAR4	6	2	33
SPAR5	5	2	40
SPAR6	6	5	83
SPAR7	7	3	43
SPAR8	6	5	83
SPAR10	5	3	60
SPAR11	8	4	50
SPAR18	6	4	67
SPAR23	2	1	50
SPAR27	4	2	50
SPAR28	2	1	50
SPAR30	2	2	100
SPAR31	7	5	71
SPAR34	7	5	71
SPAR37	4	2	50
SPAR38	2	1	50
SPAR41	8	6	75
SPAR43	9	5	56
Total	120	68	-
Mean	5	2.9	56.67

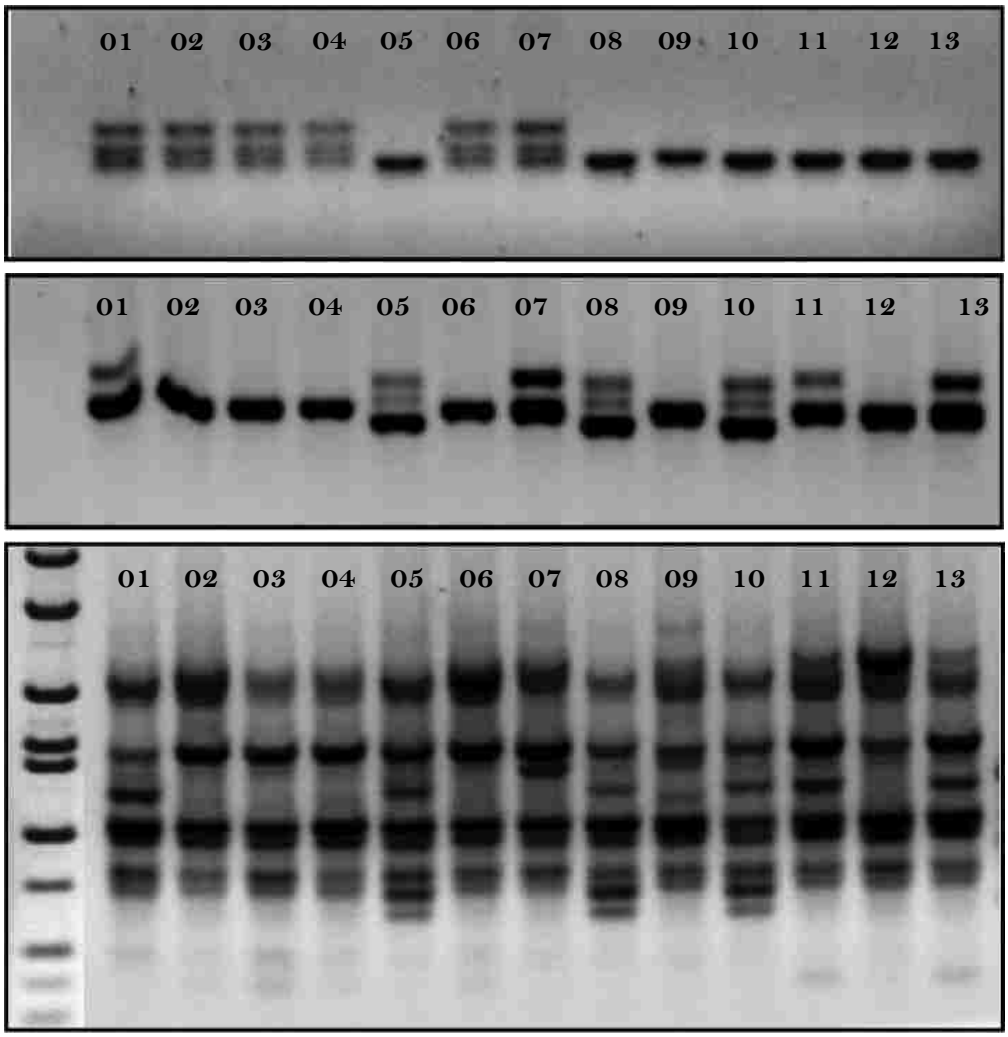


Figure 2.1. Representative gels showing fragment amplification from the 13 smooth cordgrass accessions by gSSR (Spar4; upper), eSSR (eSSR58; middle), and RAPD (OPA1; bottom) primers. Lane 1-13 = CP1-CP13 as in Table 2.1.

the accessions with a maximum number (9 and 12) of specific loci by seven (OPA1, OPA2, OPA4, OPA18, OPA19, OPB12 and OPW19) and eight (OPA1, OPA2, OPA3, OPT17, OPX3, OPX4, OPX15 and OPW19) primers, respectively, while three accessions (CP1, CP3 and CP10) were distinguished by three primers each (OPA16, OPX4, OPX15; OPB3, OPB11, OPT17; and OPA16, OPA18 and OPB12). The rest four accessions (CP4, CP5, CP8 and CP13) amplified specific loci using only a single primer (OPA3, OPW19, OPB3 and OPB10, respectively (Table 2.6).

Of the twenty-three polymorphic SSR primers, 10 (eSSR29, SPAR6, SPAR7, SPAR8, SPAR28, SPAR31, SPAR34, SPAR38, SPAR41 and SPAR43) amplified loci specific to five CP accessions (CP1, CP7, CP9, CP10 and CP12; Table 2.7). Six primers (eSSR29, SPAR6, SPAR7, SPAR28, SPAR31 and SPAR38) amplified an allele specific by four accessions (CP9, CP7, CP12, CP10). SPAR 34 produced alleles that are specific to CP1, CP7, whereas SPAR8 and SPAR41 amplified identical alleles for CP9 and CP12. SPAR43 amplified three alleles that were common to CP1, CP9, and CP12 (Table 2.7). Three (CP7, CP9 and CP12) were separated from the rest of the accessions by amplifying the maximum number (4) of specific loci by four primers (SPAR6, SPAR7, SPAR34, SPAR38; eSSR29, SPAR8, SPAR41, SPAR43; and SPAR8, SPAR28, SPAR41 and SPAR43, respectively), while an allele specific to CP10 was amplified by a SSR (SPAR31) primer (Table 2.7).

2.3.3 Genetic Similarity Based on RAPD and SSR Markers

The genetic similarities (GS) values, based on Jaccard's coefficients, were slightly different for RAPD and SSR markers among 13 accessions of smooth cordgrass. GS_{RAPD} ranged from 0.09 to 0.74. The similarity matrix indicated that the lowest genetic similarity (0.09) was between the accessions CP7 and CP9, and the highest similarity (0.74) was between CP13 and CP11 (Table 2.8a). GS_{SSR} ranged from 0.06 to 1.00. The maximum genetic similarity (1.00) observed by SSR was between CP3 and CP2, CP6 and CP4, and CP11 and CP13, while the lowest genetic similarity of 0.06 was between CP9 and CP2, CP9 and CP3, and CP9 and CP5 (Table 2.8b). $GS_{RAPD/SSR}$ ranged from 0.11 to 0.79 (Table 2.8c), with the maximum genetic similarity (0.79) between CP11 and CP13, and the lowest genetic similarity (0.11) between CP4 and

CP9, and CP7 and CP10. GS_{SSR} of the five individual plants from each of the 13 accessions of smooth cordgrass ranged from 0.03 to 1.00. The similarity matrix indicated that the lowest GS (0.03) was between CP9 and CP2, and CP9 and CP3, while the maximum GS observed by SSR was (1.00) between CP2 and CP3, CP2 and CP6, and CP3 and CP6. The Mantel test resulted in a statistically significant positive correlation coefficient (r = 0.86) between the similarity matrices produced by the RAPD and SSR markers.

2.3.4 Cluster Analysis of the Smooth Cordgrass Accessions

Genetic similarity values generated by the RAPD and SSR polymorphic markers were utilized to study and understand the genetic relationships among the 13 accessions of smooth cordgrass by cluster analysis using the UPGMA method (Figure 2.2). The cluster results indicated that the relationships among the accessions revealed by RAPD markers were slightly different from that revealed by the SSR markers. The cluster analysis of the accessions with RAPD markers (208 loci) resulted in four clusters at the GS ≥ 0.19 (Figure 2.2). Five accessions (CP1, CP5, CP8, CP10 and CP9) formed one Cluster (Cluster I); three accessions (CP11, CP13, and CP12) formed Cluster II; four accessions (CP2, CP3, CP6, and CP4) formed Cluster III; and CP7 was the lone member of Cluster IV. It can be observed from the dendrogram that within Cluster II, CP11 and CP13 appeared to be closer to each other, with a similarity coefficient of 0.74 In addition, it can be noted that CP7 and CP9 were the most diverse accessions within the cluster analysis, with a similarity coefficient of 0.09.

Cluster analysis with SSR markers (68 Loci) separated the 13 accessions into four Clusters (Figure 2.2). Five accessions (CP1, CP2, CP3, CP4, and CP6) formed the Cluster I. Cluster II (CP7, CP9 and CP12) and Cluster III (CP5, CP8 and CP10) consisted of three accessions each; and Cluster IV included CP11 and CP13.

The RAPD and SSR data obtained for all 276 polymorphic loci were combined to estimate genetic similarity and for UPGMA cluster analysis among the 13 accessions. The Jaccard's similarity coefficient values ranged from 0.11 to 0.79, and as seen in the dendrogram the accessions were separated into five main clusters at GS > 0.20 (Figure 2.2). Cluster I was comprised of five accessions CP1, CP2, CP3, CP4, and CP6. Cluster

Table 2.6. Identification of accession-specific allele (unique tags) by RAPD primers

RAPD markers	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13
OPA1							✓		✓			✓	
OPA2							✓		✓			✓	
OPA3				✓					✓				
OPA4							✓						
OPA13												✓	
OPA16	✓									✓		✓	
OPA18							✓			✓			
OPA19							✓					✓	
OPB3			✓					✓					
OPB10													✓
OPB11			✓										
OPB12							✓			✓			
OPT17			✓						✓				
OPX3									✓			✓	
OPX4	✓								✓				
OPX15	✓								✓				
OPW19					✓		✓		✓				

Table 2.7. Identification of accession-specific allele (unique tags) by SSR primers

SSR markers	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13
eSSR29									✓				
SPAR6							✓						
SPAR7							✓						
SPAR8									✓			✓	
SPAR28												✓	
SPAR31										✓			
SPAR34	✓						✓						
SPAR38							✓						
SPAR41									✓			✓	
SPAR43	✓								✓			✓	

Table 2.8a. Jaccard's similarity coefficient matrix of the 13 smooth cordgrass accessions based on the RAPD markers

	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13
CP1	1.00												
CP2	0.33	1.00											
CP3	0.32	0.55	1.00										
CP4	0.17	0.38	0.40	1.00									
CP5	0.30	0.19	0.23	0.22	1.00								
CP6	0.18	0.52	0.47	0.49	0.21	1.00							
CP7	0.18	0.23	0.17	0.16	0.19	0.31	1.00						
CP8	0.30	0.21	0.22	0.20	0.68	0.24	0.20	1.00					
CP9	0.23	0.18	0.16	0.11	0.24	0.13	0.09	0.21	1.00				
CP10	0.23	0.13	0.21	0.14	0.50	0.15	0.10	0.50	0.26	1.00			
CP11	0.19	0.22	0.21	0.18	0.23	0.20	0.15	0.28	0.24	0.35	1.00		
CP12	0.16	0.19	0.23	0.19	0.19	0.26	0.19	0.20	0.23	0.28	0.36	1.00	
CP13	0.15	0.17	0.18	0.16	0.19	0.16	0.13	0.20	0.21	0.29	0.74	0.36	1.00

Table 2.8b. Jaccard's similarity matrix coefficient of the 13 smooth cordgrass accessions based on the SSR markers

	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13
CP1	1.00												
CP2	0.29	1.00											
CP3	0.29	1.00	1.00										
CP4	0.24	0.89	0.89	1.00									
CP5	0.19	0.12	0.12	0.12	1.00								
CP6	0.24	0.89	0.89	1.00	0.12	1.00							
CP7	0.16	0.09	0.09	0.12	0.12	0.12	1.00						
CP8	0.16	0.12	0.12	0.12	0.90	0.12	0.12	1.00					
CP9	0.13	0.06	0.06	0.10	0.06	0.10	0.21	0.09	1.00				
CP10	0.16	0.12	0.12	0.12	0.81	0.12	0.12	0.90	0.09	1.00			
CP11	0.12	0.09	0.09	0.09	0.15	0.09	0.15	0.19	0.13	0.19	1.00		
CP12	0.17	0.21	0.21	0.17	0.09	0.17	0.20	0.09	0.32	0.09	0.09	1.00	
CP13	0.12	0.09	0.09	0.09	0.15	0.09	0.15	0.19	0.13	0.19	1.00	0.09	1.00

Table 2.8c. Jaccard's similarity coefficient matrix of the 13 smooth cordgrass accessions based on SSR+RAPD markers

	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13
CP1	1.00												
CP2	0.32	1.00											
CP3	0.31	0.63	1.00										
CP4	0.19	0.48	0.49	1.00									
CP5	0.28	0.17	0.20	0.19	1.00								
CP6	0.19	0.59	0.55	0.59	0.19	1.00							
CP7	0.18	0.19	0.15	0.15	0.17	0.26	1.00						
CP8	0.27	0.19	0.20	0.18	0.72	0.21	0.18	1.00					
CP9	0.21	0.15	0.14	0.11	0.19	0.13	0.12	0.18	1.00				
CP10	0.21	0.13	0.19	0.14	0.56	0.15	0.11	0.57	0.22	1.00			
CP11	0.17	0.19	0.18	0.16	0.21	0.17	0.15	0.26	0.22	0.31	1.00		
CP12	0.16	0.19	0.22	0.18	0.16	0.24	0.19	0.18	0.25	0.23	0.29	1.00	
CP13	0.14	0.15	0.16	0.14	0.18	0.14	0.14	0.20	0.19	0.26	0.79	0.30	1.00

II comprised one accession CP7. Cluster III (CP5, CP8, and CP10) and Cluster IV (CP11, CP13, and CP12) comprised of three accessions each. Cluster V was formed by a single accession CP9, which showed low similarity (GS ~0.20) with other accessions.

Cluster analysis with the fingerprint data of 5 individual plants within each of the 13 accessions also resulted in four clusters at GS > 0.20. Four accessions, CP1, CP2, CP3, and CP6, formed Cluster I. Cluster II (CP5, CP8 and CP10), Cluster III (CP4, CP12, and CP7), and Cluster IV (CP9, CP11 and CP13) was composed of three accessions each. Accessions CP2, CP3, and CP6 were genetically identical with a similarity coefficient of 1.00.

The clustering pattern of the accessions, genotyped with only SSR markers, was similar to that when SSR and RAPD markers were combined together. However, the RAPDs markers generated a slightly different dendrogram with some variation in the clustering of smooth cordgrass accessions. The correlation between the matrices of cophenetic correlation values was used to check the goodness of fit of the cluster analysis. The goodness of fit of the UPGMA dendrogram was confirmed by the highly significant correlation values for RAPD (r = 0.93), SSR (r = 0.99), and RAPD+SSR (r = 0.97). The statistically significant, high cophenetic correlation coefficients (r) validated a good representation of the relationship between the dendrogram clusters and the similarity matrices.

In order to generate a more comprehensive representation of the relationships among the accessions, a principal coordinate analysis (PCoA) was performed with the genetic similarity matrices generated by RAPD, SSR and RAPD+SSR markers in smooth cordgrass. PCoA separated the accessions into groups similar to the ones obtained with UPGMA cluster analysis (Figure 2.3). The three coordinates accounted for 7.13 % of the variation with RAPD, 8.87 % with SSR, and 7.37 % with RAPD+SSR markers. 29.87 % variation was observed among the five individual plants within each of the 13 accessions as resolved through the SSR markers.

2.3.5 Analysis of Molecular Variance

Analysis of molecular variance (AMOVA) was performed to assess the variation among and within the smooth cordgrass accessions, using the genetic distance matrix

generated by SSR markers. Partitioning of the total variation by AMOVA indicated that SSR markers explained for 38 % (F_{ST} = 0.38) of the total genetic variations attributed among 13 smooth cordgrass accessions. Conversely, 62 % of the variation was explained by the differences among individual plants (Table 2.9). These variation components were highly significant (P<0.001) based on the permutation test, and it suggests the presence of enough genetic variation within the accessions for further exploitation in breeding program.

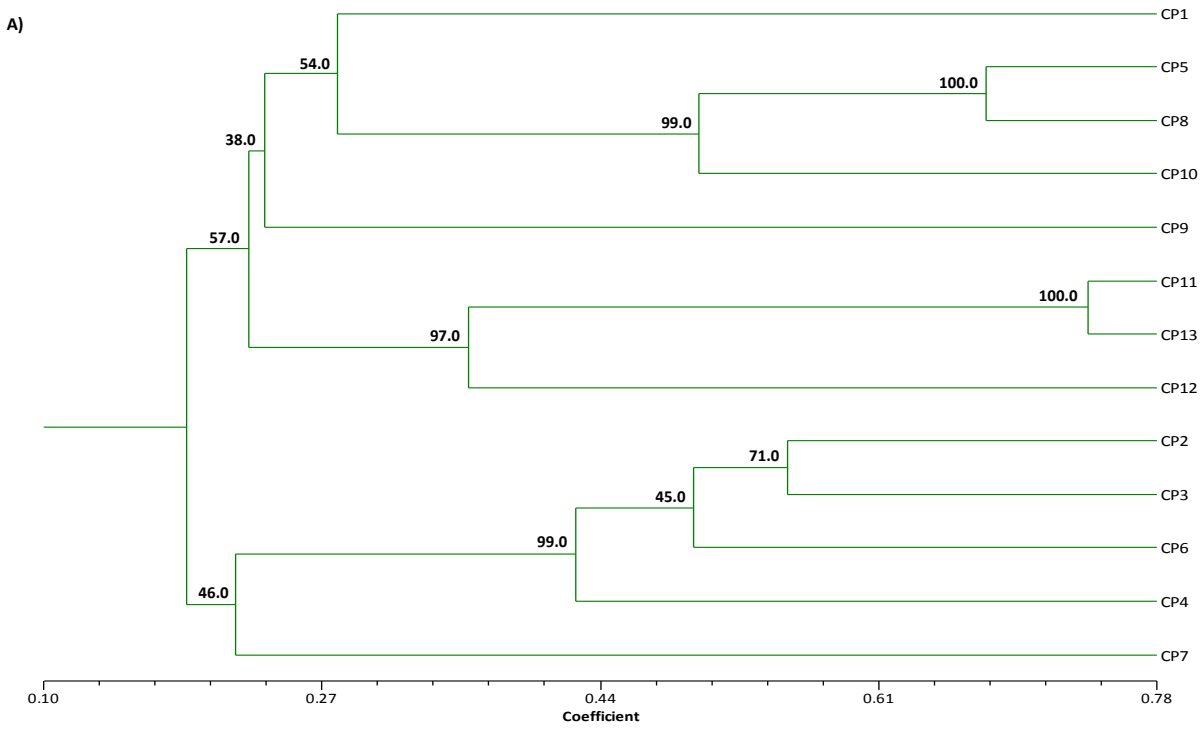
2.4 Discussion

Evaluation of the genetic diversity/relatedness within a breeding germplasm pool has become crucial and DNA markers are central to germplasm characterization and conservation, and long-term management that require an understanding of the genetic structure of a population (Zaghloul et al., 2006). The extent of genetic diversity in a species determines its ability to adapt to changing environmental challenges (Anand et al., 2004). Smooth cordgrass has a potential to quickly adapt to different ecology systems and therefore is considered an environmental engineer helping in coastal erosion control and marsh restoration (Utomo et al., 2009).

Different marker systems, by virtue of their unique properties, can reflect various aspects of genetic diversity (Karp and Edwards, 1997). In this research, two different marker systems, RAPD and SSR, were used to compare and define genetic relationships among 13 smooth cordgrass accessions.

2.4.1 Polymorphism in Smooth Cordgrass

Genotyping data with 13 smooth cordgrass accessions indicated that a higher proportion of RAPD primers were polymorphic than the SSR primers and the RAPD primers produced higher average number (9.3) of alleles/primer compared to SSR (2.4). However, the percentage of the polymorphic fragments and the PIC values of the RAPD and SSR polymorphic markers were not significantly different (Table 2.3). Although the 13 smooth cordgrass accessions have their origin in different Parishes of Louisiana (Table 2.1), the relatively low level of polymorphism among them may be due to the fact that



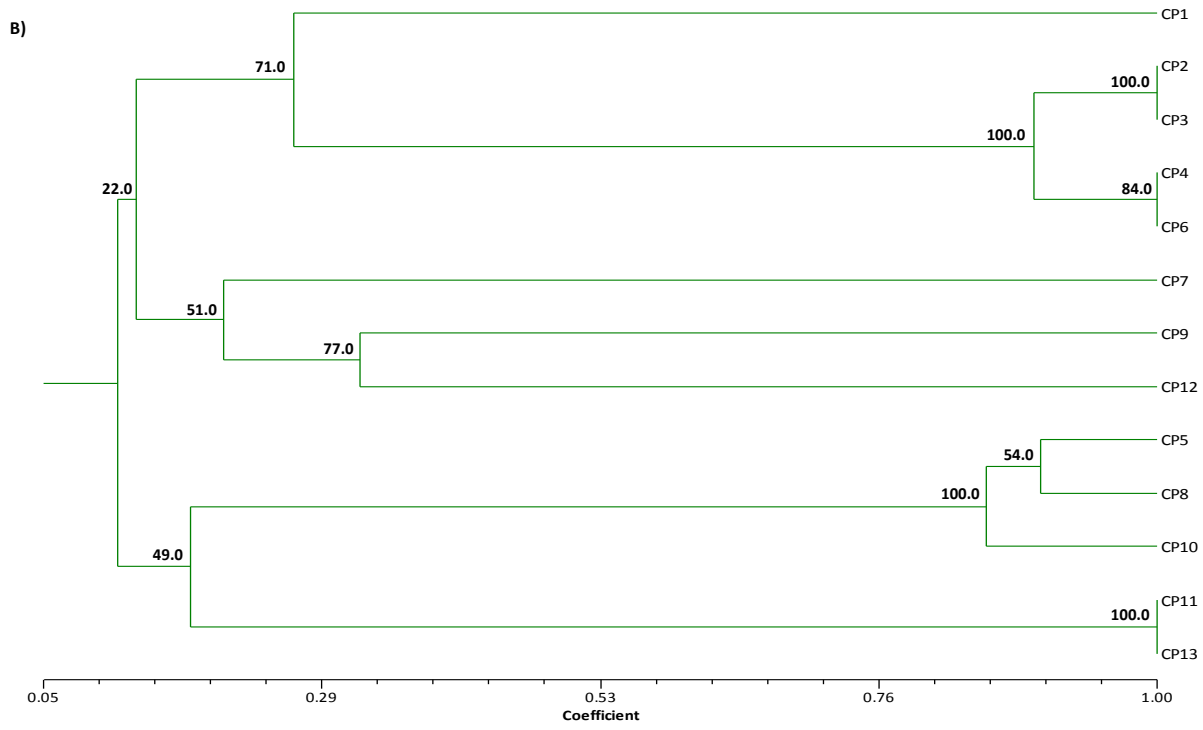


Figure 2.2. Cluster analysis of the 13 smooth cordgrass accessions with UPGMA based on Jaccard's coefficient. A) Dendrogram for RAPD markers, B) Dendrogram for SSR markers.

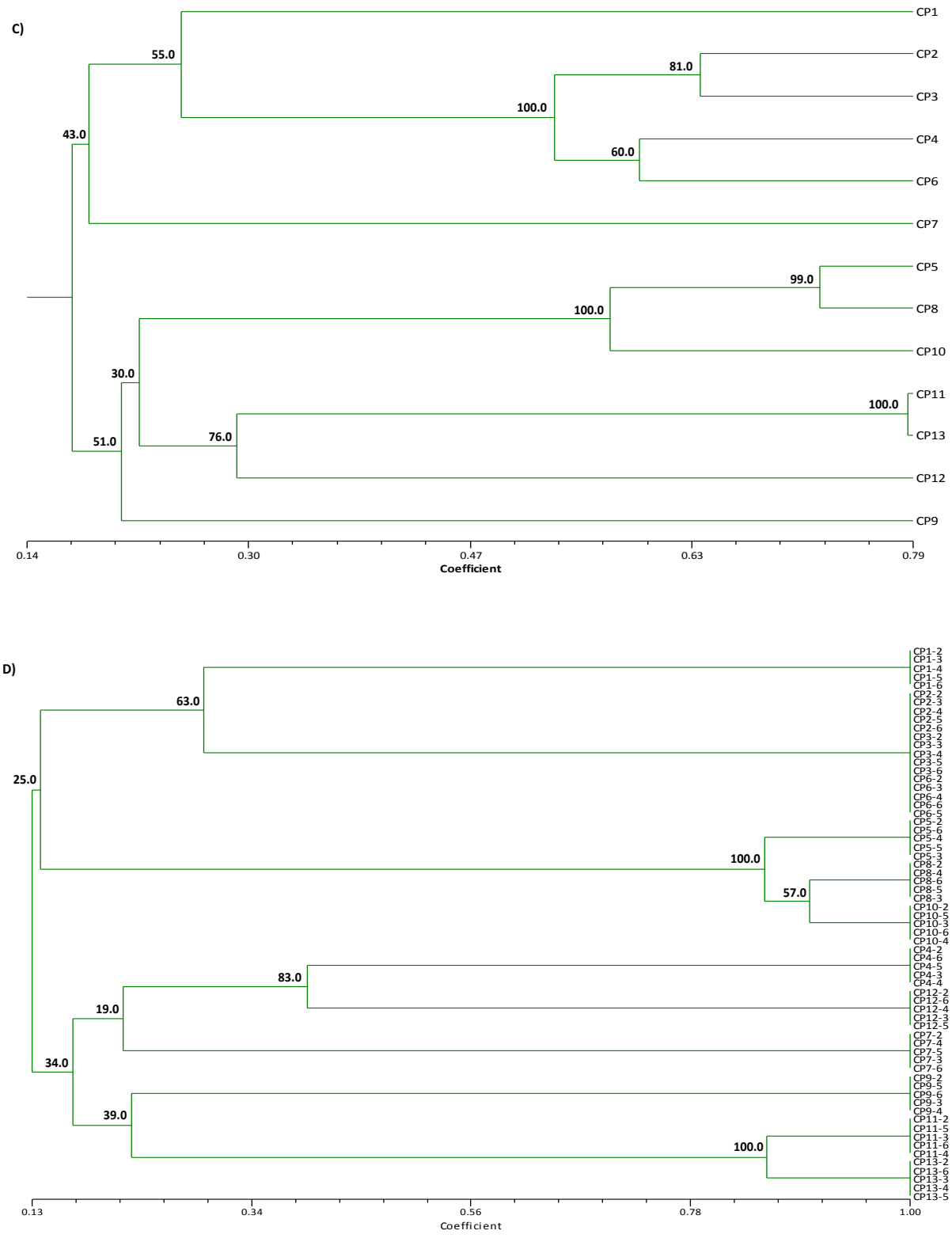


Figure 2.2. (Cont.). Cluster analysis of the 13 smooth cordgrass accessions with UPGMA based on Jaccard's coefficient. C) Dendrogram for RAPD+SSRs, and D) Dendrogram for five individual plants within each of the 13 accessions.

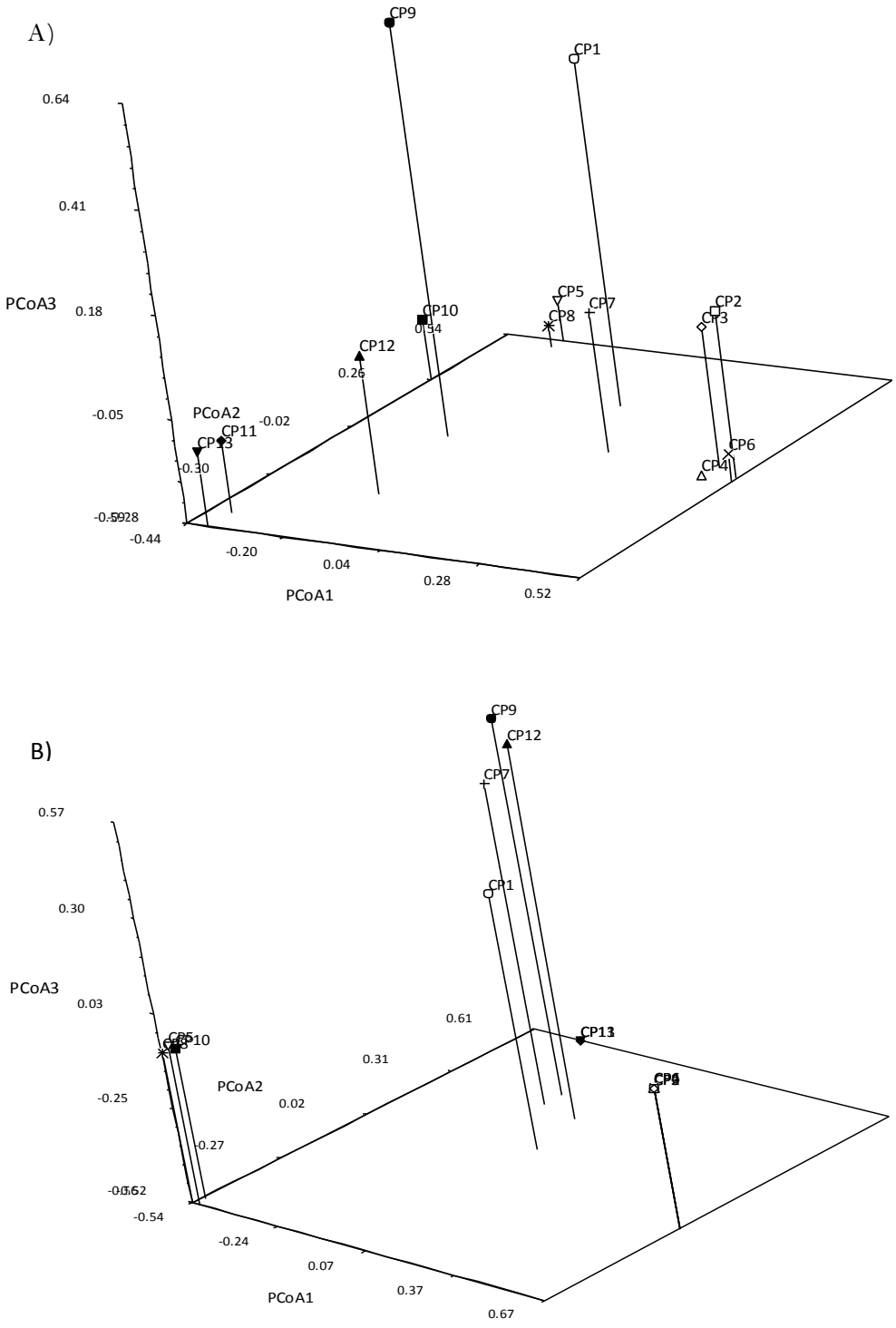


Figure 2.3. Three dimensional principal coordinate analysis (PCoA) plot of the 13 smooth cordgrass accessions based on RAPD (A), SSR (B) makers.

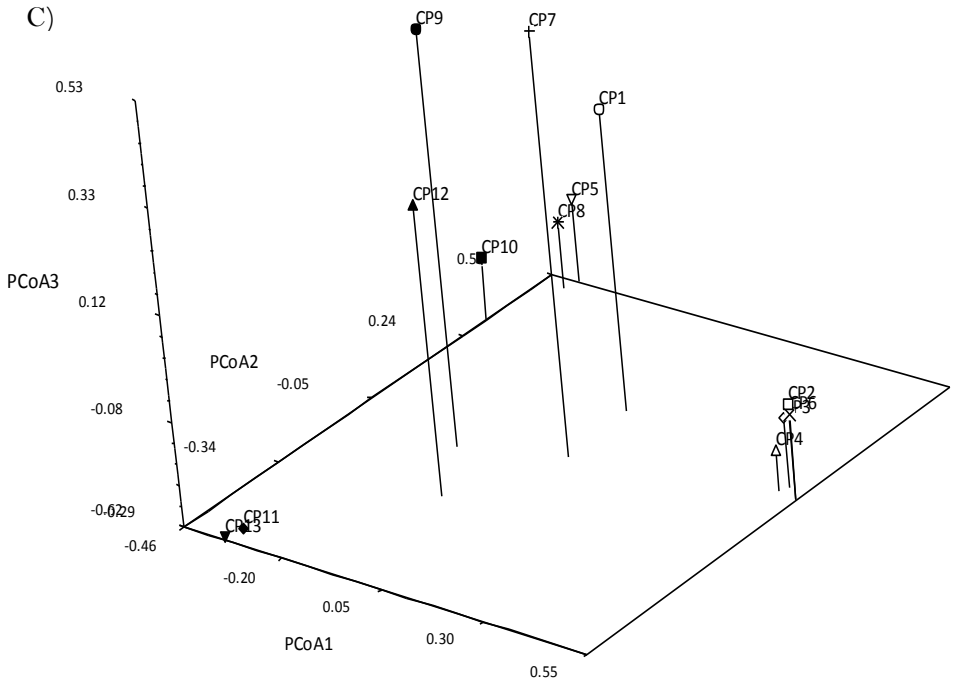


Figure 2.3. (Cont.). Three dimensional principal coordinate analysis (PCoA) plot of the 13 smooth cordgrass accessions based on RAPD+SSR (C) makers.

Table 2.9. Analysis of molecular variance (AMOVA) for the 13 smooth cordgrass accessions

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	3	10.096	0.18931 Va	38.11
Within populations	61	18.75	0.30738 Vb	61.89
Total	64	28.846	0.49668	
Fixation Index	FST :	0.38114		

d.f., degree of freedom;

they are closely related where natural polycross (intercross-breeding) events could occur, resulting in development of new genotypes. In such a case, the genetic diversity is not usually conserved (Wu et al., 2005). Despite low overall proportion of diversity among the accessions, RAPD and SSR markers generated different clustering patterns, which may reflect diverse rearrangements leading to different genetic variation among the accessions at the loci that were targeted by the two marker systems. RAPD markers are effective in revealing genetic variability among the elite accessions because of their wider coverage in the genome, while SSR markers possess hypervariability at pre-determined repetitive sites of DNA (Cuesta et al., 2010), but both markers had high resolving power in the present study. There are no earlier reports comparing both marker systems in smooth cordgrass, but these techniques have been individually evaluated in previous studies (Ryan et al., 2007; Baisakh et al., 2009) that showed polymorphism levels comparable with the present results (Ryan, 2003). The polymorphism rate observed in the present study (57.3 %) was slightly higher than reported by Stiller and Denton (1995) where 41 % (29 RAPD fragments) polymorphism was observed using 40 primer sets. However, O'Brien and Freshwater (1999) found 75 % (225 bands) polymorphism among five individual plants from each of five geographic areas along the Atlantic and Gulf coasts of the United States, while, Ryan (2003) could detect even higher (86 %; 174 bands) rate of polymorphism with 14 RAPD primers.

In this study 37.7 % (23 out of the 61) of the SSR primer pairs generated polymorphic fragments in smooth cordgrass, which was higher than that reported for other plant species such as wheat (25 %; Eujayl et al., 2002), barley (35 %; Thiel et al., 2003), soybean (12. 8 %; Han et al., 2004) and cotton (18.2 %; Hisano et al., 2007). However the SSR primers detected lower polymorphic loci in smooth cordgrass, which corroborates the finding of a previous study by Baisakh et al. (2009) who also reported lower polymorphic loci (22.2 %) in the same species. The low resolution of the SSR primers for polymorphic loci may be attributed to the interrelationship of the accessions, the small number of markers and accessions used, and the hexaploid nature of smooth cordgrass (Travis et al., 2002). As expected the genetic similarity value among the smooth cordgrass accessions explained by the RAPD markers (mean GS = 0.32) was lower than that by the SSR markers (mean GS = 0.45). The SSR primers generated less number of loci (an average of 2.96 per primer) compared to the RAPD primers with an

average 6.5 loci/primer in the 13 smooth cordgrass accessions. This further justified the lower levels of polymorphism revealed by the SSRs in comparison to the RAPD markers. The low level of polymorphism by the SSRs, in general, could be due to the eSSRs used, which target the variation within the otherwise conserved coding region of a gene (Eujayl et al., 2002; Sen et al., 2010; Wen et al., 2010). However, the eSSR markers will be of great significance and value to genetic association studies of smooth cordgrass with regard to agronomically desirable/superior traits. Similar to the present finding that the RAPD primers were more polymorphic than the SSR primers, Chimote et al. (2007) and Leal et al. (2010) demonstrated earlier that the RAPD primers produced more polymorphism information than the SSR markers in potato. On the other hand, the SSR markers could resolve more diversity than the RAPD markers in maize (Cholastova et al., 2011). Overall, these results indicated that the level of DNA variation observed with the two marker systems was sufficiently informative to assess the genetic variability among the 13 smooth cordgrass breeding lines.

2.4.2 UPGMA Clustering

Cluster analysis provided a better resolution of the genetic relationship among the 13 smooth cordgrass accessions, used in this study, from Southern Louisiana. The RAPD and the SSR markers grouped the 13 accessions into four major clusters but with some differences in their member assignment (Figure 2.2), whereas both markers, when combined, produced five clusters. Dendrograms showed that CP7 and CP9 were the most diverse accessions. Clustering pattern, however, did not show grouping of the accessions according to their geographical site of collection. Nonetheless, higher level of similarity was observed in smooth cordgrass accessions that originated from the same or close parishes (region). Accessions from Lafourche (CP1, CP2 and CP3) remained closely clustered, as did Cameron (CP4) and Terrebonne (CP6), and Cameron (CP5) and Vermillion (CP8). CP11 and CP13 were clustered in a separate group, probably because of their origin from cross-pollination events among the accessions CP1 to CP8. The shift in cluster pattern attributed to the levels of diversity found in CP7, CP9 and CP12 is possible because of the outcrossing (Fang et al., 2004) and polyploid nature of smooth cordgrass. Cross-pollination events would allow development of new accessions with

heterogeneous genome expanding the variation of genotypic progeny (Ryan et al., 2007). Principal coordinate analysis (PCoA) is expected to be more informative about genetic differentiation of accessions among groups. In the present study, the results from the PCoA corroborated the results from the cluster analysis i.e., same groups were identified by the 3D principal components and UPGMA clustering. The findings revealed that the 13 smooth cordgrass accessions were tightly distributed across the plain and an association was detected between the genetic clustering and the parish from which they were collected (Figure 2.3).

The cophenetic correlation between the similarity matrix and corresponding dendrogram for both the SSR (r = 0.99) and the RAPD (r = 0.93) markers revealed a very high degree of fit, which is possibly due to the moderate number of pair-wise genetic similarity coefficients with extreme values. This was further validated by the bootstrap analysis, where the node connecting Cluster I and Cluster II of the SSR dendrogram appeared in only 22 % bootstrap steps. The close similarity of the clustering pattern by the RAPD and the SSR markers was evident from the Mantel test that showed a significant correlation between the two marker systems (r = 0.86; r^2 = 0.74). This suggested that the two marker systems captured the genetic variation almost similarly among the smooth cordgrass accessions.

Through this investigation, unique RAPD and SSR marker tags (ID) were identified for 10 and 5 accessions, respectively. These 'tags' in smooth cordgrass accessions will be very useful for their genetic identify. Detail analysis with more number of primer pairs would be necessary to generate more reliable genetic profiles and unique genotypic ID for all accessions, although Blum et al. (2007) were of the opinion that 10 genetic markers would be sufficient to differentiate smooth cordgrass accessions. Although most primers amplified alleles common to a set of accessions, any two accessions could be easily distinguished by the use of one or more primers.

2.4.3 AMOVA Analysis

Analysis of molecular variance (AMOVA) revealed higher genetic variation within groups in contrast to the lower levels of genetic differentiation among the accessions. Earlier studies (O'Brien et al., 1999; Travis et al., 2002; Fisher et al., 2005; Blum et al.,

2007; Ryan et al., 2007; Utomo et al., 2009; Novy et al., 2010) have also documented similar results for smooth cordgrass populations and they concluded that polycross events in the field may result in the establishment of novel genotypes affecting genetic diversity among and within populations. Because smooth cordgrass is an open pollinated plant, it is possible that greater genetic exchange occurs among individuals within a group or a study area (Utomo et al., 2009). The genetic diversity among the accessions is sufficiently large to allow exploitation in the breeding programs aimed at development of agronomically superior cultivars.

Chapter III
Differential Expression of Genes and Genetic Diversity for Cold Stress Response of Sugarcane (*Saccharum* spp Hybrids)

3.1 Introduction

Sugarcane (*Saccharum* spp hybrids), like other agricultural plants, has been grown in many parts of the world for different purposes. It is used to satisfy human and animal needs such as food, feed, fiber, fuel, and construction materials (D'Hont et al., 1998). Sugarcane, being the main sugar-producing crop in the world, is considered an important source of protein, carbohydrates and other nutrients. Sugarcane is a non-cereal grass cultivated widely in tropical and subtropical regions of the world primarily for sugar and sugar-derived ethanol, and recently has gained particular attention as a second generation energy crop for cellulosic ethanol due to its high-biomass (Suman et al., 2011; Pinto et al., 2010).

3.1.1 Taxonomy of Sugarcane

Sugarcane belongs to the complex genus *Saccharum* for its high degree of polyploidy and interspecific origin (D'Hont et al., 1998). The *Saccharum* genus falls within the grass family, Poaceae (Table 3.1; D'Hont et al., 1998), which also includes other tropical grasses such as sorghum and maize (corn). *Saccharum* is composed of at least six distinct species that includes *S. spontaneum*, *S. robustum*, *S. officinarum*, *S. barberi*, *S. sinense*, and *S. edule* (Table 3.2; D'Hont et al., 1998). *Saccharum spontaneum*, also known as wild cane, is a highly polymorphic with variable chromosome number (2n=40 to 128). This is a disease-resistant species that produces little sugar and is also highly adapted to cold and drought stress. On the other hand, *Saccharum officinarum*, otherwise known as the noble cane, is a domesticated species with high sugar content and its chromosome number is 2n=80 (Cordeiro et al., 2007). Modern cultivars of sugarcane (*Saccharum* spp.) are hybrid complexes derived from crosses between *S. officinarum* and *S. spontaneum* (Edmé et al.,

2006). Commercial breeding programmes have mainly used these two major progenitors to study their genetic variation. In the early 1900s, these programmes were focused on interspecific crosses involving *S. officinarum* and *S. spontaneum*. This led to incorporation of valuable agronomic traits, such as disease resistance, but also required a backcrossing programme with *S. officinarum* to raise the sucrose content in a process called "nobilization" (Stevenson, 1965). Sugarcane (interspecific hybrids of *Saccharum*) is thought to have originated from Asia and then brought to the Western Hemisphere by Columbus (Edmé et al., 2005).

Table 3.1. Taxonomy of sugarcane

Kingdom : Plantae (plants)
Phylum : Magnoliophyta (flowering plants)
Class : Liliopsida (monocotyledons)
Order : Cyperales (grasses, sedges)
Family : Poaceae (grasses and bamboos)
Genus : *Saccharum*
Species : *Saccharum officinarum* (sugarcane)

Table 3.2. Summary of the members of the genus *Saccharum*

Species	Classification	Sugar content	Chromosome number
S. barberi	Ancient hybrid	Low	2n=11-120
S. edule	Wild species	Used as a vegetable	2n=60-80
S. officinarum	Noble canes	High	2n=80
S. robustum	Wild species	Nil	2n=60-~200
S. sinense	Ancient hybrid	Low	2n=80-140
S. spontaneum	Wild species	Nil	2n=40-128

3.1.2 Economic Importance

Sugarcane is the leading farm crop in Louisiana agriculture. Worldwide, sugarcane is the main crop with an annual production of 1.69 billion tonnes of sugarcane stalks (FAOSTAT, 2011; http://faostat.fao.org/). Sugarcane productivity differs widely among different countries. USA occupies 11th place in sugarcane production (25 million tonnes). Brazil is the largest producer with 719 million tonnes harvest in 2010 followed by India (278 million tones), China (111 million tones), and Thailand (68 million tonnes harvest; FAOSTAT, 2011). In USA, sugarcane occupies 5th place after maize, soybean, wheat and sugar beet, with an annual production of more than 24 millions tons on 350 Ha (FAOSTAT, 2011). In the United States, sugarcane is commercially grown in Florida, Louisiana, Hawaii, and Texas. In Louisiana, sugarcane is grown in 23 Parishes with an annual production of 14,000 tons with a cane yield ranging from 30 to 35 tons per acre (NASS, 2011; http://www.nass.usda.gov/).

3.1.3 Cold Tolerance

The development and physiology of a plant is a well-coordinated program of gene expression as a result of the interaction of its genome and its internal and external environment (Kauffman et al., 2010). Sugarcane, being a (sub)tropical species, is sensitive to low temperature (below 10 ^{o}C) and its growth and yield is affected when the growing period coincides with a brief/prolonged cold period. Cold stress is one of the serious abiotic factors that can cause adverse effects on the growth, productivity and geographical distribution of crops such as sugarcane (Menossi et al., 2008). Because sugarcane is normally grown in warm climate, it may not have developed the tactics to avoid the effects of cold (Thomashow, 2001). However, genetic variability exists in response to cold stress among the commercial sugarcane varieties (Du et al. 1999). In Louisiana, sugarcane growing season coincides with infrequent freezing temperatures. Therefore, Louisiana breeding program aims to incorporate cold tolerance as a valuable trait in its strategy to develop improved sugarcane varieties. Integration of cold tolerance in the sugarcane crop will allow expansion of its cultivation to colder regions.

3.1.4 Gene Expression Profiling

Differential gene expression is considered to be critical for the coordinated development of new cells and tissues at a specific time, which is usually identified by comparing cDNA copies of the steady-state mRNA populations under different conditions (Casu et al., 2005). These cDNAs otherwise known as expressed sequence tags (ESTs) represent the genes functioning at a specific time and tissue (Casu et al., 2004; 2005). Several molecular strategies such as representational difference analysis (RDA; Lisitsyn et al., 1993), differential display (DD; Liang et al., 1992), serial analysis of gene expression (SAGE; Velculescu et al., 1995), suppression subtractive hybridization (SSH; Diatchenko et al., 1996), annealing control primer (ACP; Hwang et al., 2003), and cDNA microarray have allowed the identification of differentially expressed genes (DEGs) involved in stress responses.

In recent years ACP system has been used frequently to isolate DEGs in both animals and plants (Kim et al., 2004; RamanaRao et al., 2011). The ACP method is an innovative and improved tool for the specificity of PCR amplification. ACP has a unique tripartite structure that contains a regulator between two different sequences, composed of a polydeoxyinosine linker. This leads to formation of a bubble-like structure at specific annealing temperatures and maximizes PCR specificity by preventing annealing of the nontarget sequence to a template, and thus facilitates highly reproducible hybridization of the primer to the target sequence (Hwang et al., 2003; Figure 3.1).

In sugarcane, SSH, cDNA microarrays, qRT-PCR, and SUSCEST data mining have been used to study responses to cold (Nogueira et al., 2003; 2005), drought (Rocha et al., 2007), and water deficit stress (Gupta et al., 2009). Nogueira et al. (2003), using high-density filter arrays containing sugarcane ESTs, identified thirty-three genes that were induced by low temperature (4 °C) stress. Nogueira et al. (2005) identified 26 non-redundant genes encoding NAC domain proteins in sugarcane, and using RNA gel-blot analysis they found that SsNAC23 was strongly induced by chilling stress in addition to water stress and herbivory. The identification of genes regulated under cold-stress and the study of their functional importance is necessary to develop cold-tolerant varieties. With a long term goal of developing gene-based markers to facilitate the breeding of cold tolerant sugarcane cultivars, the present study was started with the following objectives.

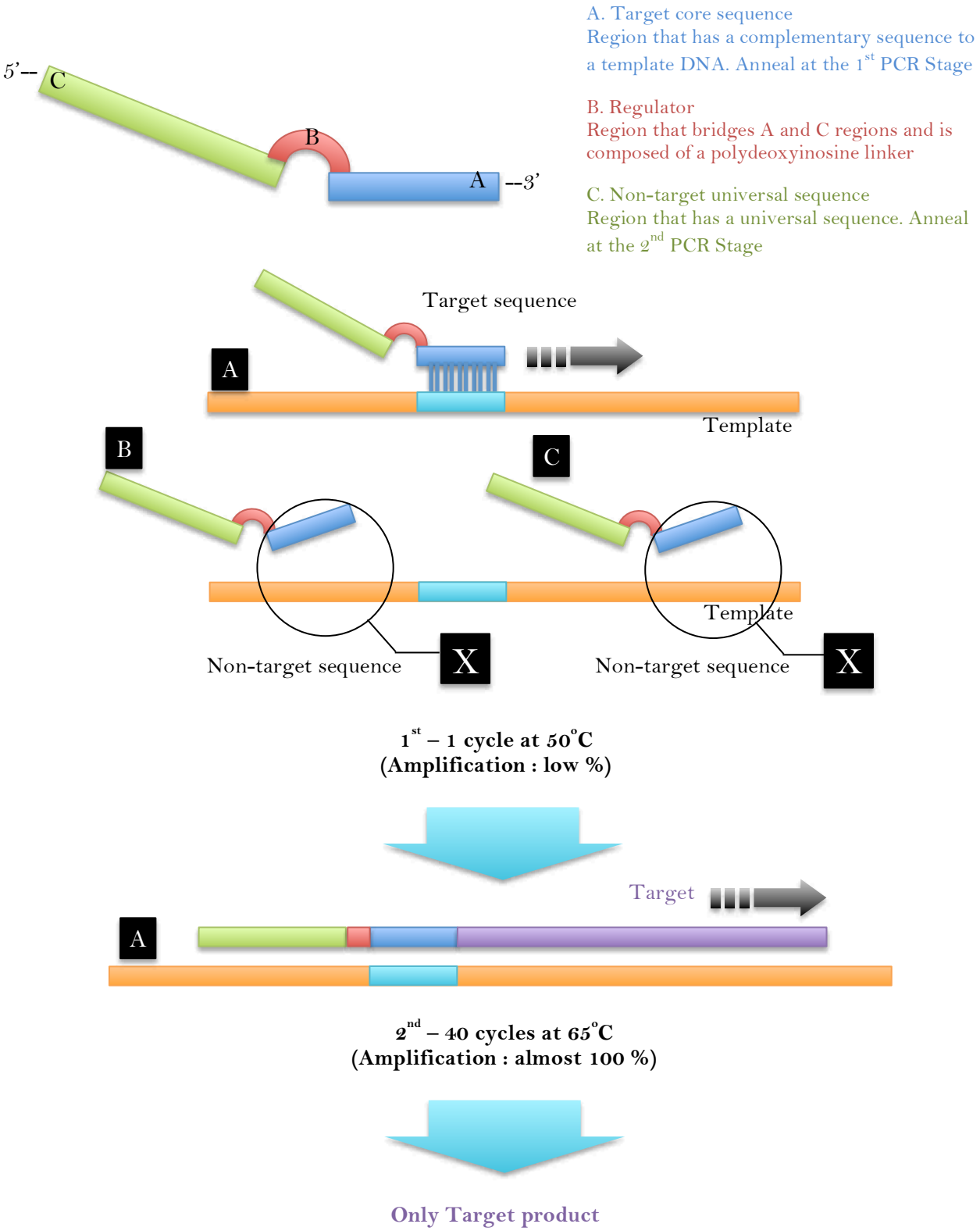


Figure 3.1. Structure of the annealing control primer (ACP) system.

3.1.5 Research Objectives

- ✤ To identify and isolate differentially expressed genes (DEGs) from a cold tolerant sugarcane clone in comparison to a cold sensitive clone through gene fishing technique;
- ✤ To identify the DEGs that are up/down-regulated under cold stress in the cold tolerant sugarcane clone;
- ✤ Study the genetic diversity among commercial sugarcane cultivars of Louisiana using SSR markers derived from known cold responsive DEGs.

3.2 Materials and Methods

3.2.1 Plant Material, Treatment and RNA Isolation

Two sugarcane cultivars, which have previously been classified as tolerant (Ho02-144) and sensitive (L79-1002) to cold stress, were used in this study. First-stubble plants were established in the greenhouse at the Sugar Research Station, St. Gabriel, LA. One month-old stubbles were exposed to cold stress during one week at a temperature of 0 ^{o}C inside a growth chamber (Shel Lab, Cornelius, OR) maintained at 14 h day/10 h night cycle. Control plants (no cold stress) were maintained in the greenhouse under natural light regime. Young leaf samples were collected from both clones at different time intervals 0 h (control), 24 h, 48 h, and 72 h after initiation of cold stress. All tissue samples were immediately frozen in liquid nitrogen and stored at -80 ^{o}C for further RNA isolation.

Total RNA was isolated from frozen leaf tissues of unstressed and cold-treated sugarcane clones using RNeasy Plant Mini Kit (Qiagen, Valencia, CA). The total RNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). An aliquot of 3 µg RNA extracted from leaf tissues at different concentrations was pooled and stored at -80 ^{o}C for use in first strand cDNA preparation.

3.2.2 First-Strand cDNA Synthesis

First-strand cDNA was synthesized from 3 µg of the pooled total RNA of cold-stressed and control leaf tissue of sensitive and tolerant clones using a Gene Fishing DEG premix kit (Seegene, Rockville, MD) as described by RamanaRao et al. (2011). Three µg of RNA was mixed with $(dT)_{15}$-ACP1 primer in a final volume of 9.5 µl and was incubated at 80 °C for 3 min followed by ice-chilling for 2 min. Reverse transcription was performed for 90 min at 42 °C in a final reaction volume of 20 µl containing 4 µl of 5X reaction buffer (Promega, Madison, WI), 5 µl of 2 mM dNTP mix, 2 µl of 10 µM cDNA synthesis primer, oligo$(dT)_{15}$-ACP1 (Seegene, Rockville, MD), 0.5 µl of RNasin RNase Inhibitor 20 U/µl (Promega, Madison, WI), and 1 µl of Moloney murine leukemia virus reverse transcriptase 200 U/µl (Promega, Madison, WI). First-strand cDNAs were 5X-diluted by the addition of 80 µl of nuclease-free water.

3.2.3 ACP-based Gene Fishing PCR

Second-strand cDNA synthesis and the next two PCR round were conducted in a single tube following RamanaRao et al. (2011). Second-strand cDNA synthesis was conducted at 50 °C (low stringency) during the first-stage PCR in a final reaction volume of 20 µl containing 3 µl (~50 ng) of the diluted first-strand cDNA, 10 µl of 2X SeeAmp ACP master mix (Seegene, Rockville, MD), 2 µl of 5 µM arbitrary ACPs (Table 3.3), and 1 µl of 10 µM oligo$(dT)_{15}$-ACP2. The first-stage PCR profile for second-strand synthesis was one cycle of 94 °C for 1 min, followed by 50 °C for 3 min, and 72 °C for 1 min. The second-stage PCR amplification profile was 40 cycles of 94 °C for 40 s, followed by 65 °C for 40 s, 72 °C for 40 s, and a 5 min final extension at 72 °C. The amplified PCR products were resolved in a 2 % agarose gel (Amresco, Solon, OH), stained with ethidium bromide, and visualized under UV and documented in a Kodak Gel Logic200 system (Carestream Health Inc, Rochester, NY). The differentially expressed genes (DEGs) were identified based on their intensity or presence/absence, and were cut and extracted from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA).

Table 3.3. Sequence of the primers used in cDNA synthesis and ACP-based PCR analysis

Use	Primer name	Sequence
cDNA synthesis primer (Reverse Transcription)	dT-ACP1	5'-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{18}$ -3'
Reverse primer (GeneFishing PCR)	dT-ACP2	5'-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$ -3'
	Arbitrary ACP2	5′-GTCTACCAGGCATTCGCTTCATXXXXXAGGCGATGCC-3′
	Arbitrary ACP5	5′-GTCTACCAGGCATTCGCTTCATXXXXXAGTGCGCTCG-3′
	Arbitrary ACP6	5′-GTCTACCAGGCATTCGCTTCATXXXXXGGCCACATCG-3′
	Arbitrary ACP7	5′-GTCTACCAGGCATTCGCTTCATXXXXXCTGCGGATCG-3′
	Arbitrary ACP8	5′-GTCTACCAGGCATTCGCTTCATXXXXXGGTCACGGAG-3′
	Arbitrary ACP9	5′-GTCTACCAGGCATTCGCTTCATXXXXXGATGCCGCTG-3′
	Arbitrary ACP10	5′-GTCTACCAGGCATTCGCTTCATXXXXXTGGTCGTGCC-3′
Forward primer (GeneFishing PCR)	Arbitrary ACP11	5′-GTCTACCAGGCATTCGCTTCATXXXXXCTGCAGGACC-3′
	Arbitrary ACP12	5′-GTCTACCAGGCATTCGCTTCATXXXXXACCGTGGACG-3′
	Arbitrary ACP13	5′-GTCTACCAGGCATTCGCTTCATXXXXXGCTTCACCGC-3′
	Arbitrary ACP14	5′-GTCTACCAGGCATTCGCTTCATXXXXXGCAAGTCGGC-3′
	Arbitrary ACP15	5′-GTCTACCAGGCATTCGCTTCATXXXXXCCACCGTGTG-3′
	Arbitrary ACP16	5′-GTCTACCAGGCATTCGCTTCATXXXXXGTCGACGGTG-3′
	Arbitrary ACP17	5′-GTCTACCAGGCATTCGCTTCATXXXXXCAAGCCCACG-3′
	Arbitrary ACP18	5′-GTCTACCAGGCATTCGCTTCATXXXXXCGGAGCATCC-3′
	Arbitrary ACP19	5′-GTCTACCAGGCATTCGCTTCATXXXXXCTCTGCGAGC-3′
	Arbitrary ACP20	5′-GTCTACCAGGCATTCGCTTCATXXXXXGACGTTGGCG-3′

ACP: Annealing Control Primer; poly(dX): polydeoxyinosine linkers; "X" represents deoxyinosine

3.2.4 Cloning and Analysis of cDNA

The DEG products were cloned using the pGEM-T Easy cloning kit (Promega, Madison, WI) following the manufacturer's instructions and the method described earlier (Baisakh et al. 2006). The ligated products were transformed into competent *Escherichia coli* DH5α cells; plated onto LB agar containing 100 µg/ml ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 2% X-gal; and incubated at 37 °C overnight. White colonies were picked up and grown overnight on LB liquid media supplemented with 100 µg/ml ampicillin. Plasmid DNA was isolated using a GeneJET™ Plasmid Miniprep Kit (Fermentas, Glen Burnie, MD) and tested for the presence and size of inserts by PCR using M13 universal primers. Plasmids with an insert size more than 300 bp were sequenced with T7 primer in an ABI 3730x1 genetic analyzer (High-Throughput Genomics Unit, Washington State University, WA).

The Vector sequence and the poly(A) tail was cleaned manually from the DNA sequences. Then, functional annotation of the DEGs was performed by comparing the sequences against the nonredundant nucleotide and protein database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLASTN and BLASTX) (http://www.ncbi.nlm.nih.gov/BLAST). Sequence match exceeding 50 nucleotides and more than 90% identity were considered significant and used for further analysis. The functional classification of the DEGs was done using online Gene Ontology (GO) resource (http://www.geneontology.org/).

3.2.5 Transcript Analysis of DEGs by Semi-quantitative Reverse Transcription Polymerase Chain Reaction

The expression pattern of five DEGs was validated by semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR) following the method described earlier (RamanaRao et al., 2011). Gene-specific primers were designed using Primer3Plus web resource (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), with the annealing temperature (Ta) of each primer optimized to 58 °C. The elongation factor gene (SoEF1α; GenBank Acc. # EF581011) from *S. officinarum* was used as an

endogenous reference gene. All the primers were synthesized by Integrated DNA technologies (IDT Inc, Coralville, IA).

First-strand cDNA was synthesized with 1 µg of the total RNA isolated from leaf tissues from the control (0 h) and cold-stressed plants of both L79-1002 and Ho02-144 at 24 h, 48 h, 72 h, using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacture's instructions. One µl of the first-strand cDNA was amplified using DEG-specific primers following the method of Baisakh et al. (2006) with the following PCR profile: initial denaturation 95 °C, 3 min; 35 cycles of 95 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min; and final extension 72 °C, 5 min. The amplification products were separated on 2 % agarose/EtBr (Amresco, Solon, OH) gels and analyzed under UV light using the Gel Logic 200 Imaging system (Kodak, New Haven, CT). The PCR was repeated twice to verify the reproducibility of the results.

3.2.6 Quantitative Reverse Transcription Polymerase Chain Reaction of DEGs

The relative abundance of five target genes, which represented up- and down-regulation in ACP-PCR, quantitative real-time RT-PCR (qRT-PCR) was performed with the same cDNA samples that were used for sqRT-PCR following the method described earlier (Baisakh et al., 2008). Real-time RT-PCR analysis was carried out in triplicate in a 20 µl final reaction volume, containing 2 µl of first-strand cDNA, 10 µl of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.4 or 0.2 µM of each primer, and nuclease-free water in a MyiQ real-time PCR analysis system (Bio-Rad, Hercules, CA). Real Time PCR cycling condition was: initial denaturation at 95 °C, 30 sec; 40 cycles of 95 °C, 10 sec, and 60 °C, 30 sec; and a melt curve analysis with 71 cycles of 60 °C, 30 sec. The relative expression of the mRNA (ratio) was calculated as ratio using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001), where $\Delta Ct = Ct_{control} - Ct_{stressed}$, and $-\Delta\Delta Ct = \Delta Ct_{reference\ gene} - \Delta Ct_{target\ gene}$. The elongation factor (SoEF1α) gene from *S. officinarum* (GenBank Acc. # EF581011) was used as an endogenous reference gene.

3.2.7 Mining of Expressed Sequence Tags and Development of eSSR Markers

The FASTA sequences of sugarcane cold responsive ESTs (Nogueira et al., 2005) were retrieved from the SUCEST (Sugarcane Expressed Sequence Tag) database (Vettore et al., 2001) and mined to find all perfect SSR motifs using SSRIT (Simple Sequence Repeat Identification Tool; http://www.gramene.org/microsat). A total of 12 primer pairs (eSSRs) flanking the SSR motifs were designed using the Primer3Plus portal (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with default parameters and a product size range of 150-200 bp. A M13 tail (CACGACGTTGTAAAACGAC) was added to the 5' end of all forward primers to facilitate labeling of products during the PCR reaction. All primers were synthesized by Integrated DNA technologies (IDT Inc, Coralville, IA).

3.2.8 Genotyping of Louisiana Sugarcane Cultivars Using eSSR Markers

Forty eight Louisiana sugarcane commercial clones, two clones of *S. officinarum* and 14 clones of *S. spontaneum* were genotyped with 12 eSSR primers derived from the genes responsive to cold tolerance. Genomic DNA isolated previously in the sugarcane genetics laboratory was used for PCR. The annealing temperature of each primer pair was determined using a temperature gradient ranging from 50 to 60 °C on a Thermocycler (BIO-RAD). PCR reactions were performed in a final volume of 10 µl containing 2 ng DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM dNTP mix, 0.4 unit of *Taq* DNA polymerase (Promega, Madison, WI), 0.75 µM of each primer, and ddH_2O. PCR conditions used were: initial denaturation at 95 °C for 5 min; 36 cycles of 95 °C for 45 sec, 50-60 °C for 45 sec, 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR products were detected by excitation of the fluorescence added during PCR reaction through IRDye700/IRDye800-labeled primers for multiplexing, and were run on a 6.5% LI-COR KB^{Plus} polyacrylamide gel using a LI-COR Model 4300 DNA analyzer (LI-COR Biosciences, Lincoln, NE). The gels were pre-run for 25 min. The PCR products were denatured at 95 °C for 5 min and then quickly cooled on ice, and 0.2 µl sample were

loaded with a Hamilton multi-channel pipette (Hamilton, Reno, NV) in 25 cm gels. The samples were electrophoresed at 1500 V, 40 W, 40 mA, and 45°C for 3 hours. Gel images were saved as TIFF (tagged image file format) files for further analysis.

3.2.9 Polymorphism and Genetic Diversity Analysis

Bands were scored for presence (1) and absence (0) in an Excel spreadsheet program. The polymorphism information content (PIC) value was calculated by using the formula: PIC = 2 fi (1- fi), where fi is the frequency of the amplified alleles (bands present), and (1- fi) is the frequency of the null alleles (including no amplification; Botstein et al., 1980). The estimate of eSSR-based genetic similarity (GS) and the cluster analysis among the 64 clones of sugarcane species was based on Jaccard's similarity coefficient (Jaccard, 1908) using the unweighted pair group method with arithmetic means (UPGMA) in the NTSYSpc version 2.2 program (Rohlf, 2005). Principal Coordinate Analysis (PCoA) was also used to validate the cluster distribution of the sugarcane clones.

3.3 Results

3.3.1 Effect of Cold Stress on *Saccharum* spp. Hybrids

The effect of cold stress was apparent in the cold tolerant (Ho092-144) and the cold sensitive (L79-1002) clone from the loss of chlorophyll (Figure 3.2). After one week under 0 °C the plants were transferred to the greenhouse under normal growth conditions. In Ho02-144 new tillers started to emerge after two weeks but the sensitive clone did not produce any new tiller inside the greenhouse. The leaves and stems of the sensitive clone withered with time leading to the death of the plant.

Figure 3.2. Picture showing the tolerant (Ho02-144) and sensitive (L79-1002) sugarcane clone growing under cold (0 °C) inside a growth chamber.

3.3.2 Differentially Expressed Genes Under Cold Stress in Two Sugarcane Clones

To study the molecular response of sugarcane to cold stress, RNA isolated from cold-treated (24 h, 48 h, 72 h) and untreated (control) leaf tissues of a cold tolerant clone (Ho02-144) and cold sensitive clone (L79-1002) were subjected to ACP analysis using 17 primers and two anchored oligo (dT) primers (Figure 3.3 and Table 3.3). A total of 27, and 13 DEG fragments showed a differential expression pattern between cold-treated and untreated plants of Ho02-144 and 79-1002, respectively. Of these forty DEGs, 11 exhibited lower signal intensity (downregulated genes) under cold-stress and the remaining twenty-nine exhibited increased signal intensity (upregulated genes), which were selected for further analysis.

3.3.3 Functional Annotation of the Differentially Expressed Genes

The sugarcane differentially expressed genes were named as SuDEGs followed by the clone number. The putative biological functions of the 29 cold-responsive SuDEGs were assigned after comparing their sequences against the GenBank database (www.nbci.nlm.nih.gov) (Table 3.4). These DEGs belonged to different gene families related to biological processes or molecular function as was determined from annotation of their gene ontology (GO; Table 3.4). Similarity search of the SuDEG sequences (Query) with those in the database (subject) revealed four different classes of genes. The first class consisted of 15 DEGs that showed matches to oxidative and cold-responsive genes that were previously identified in other organisms. These included Cyclophilin (CyP), 14-3-3-like protein (GF14-6), calcium transporter 1 (TRPV6), heat shock protein 23 (hsp23), cell division protease (FTSH-5), mitochondrial heat shock protein 60 (hsp60), a putative DEAD box ATP-dependent RNA helicase (ddx), a putative phenoloxidase activating factor (ppaf), a vitamin C defective 2 (VTC2) gene, cytochrome P450-related protein (CytP450), and NADP dehydrogenase quinone 1 (NDH1) (Table 3.4). The second class included four DEGs, which were not previously designated as being expressed under cold stress. Among these were a thylakoid assembly4 protein (THA4), which is involved in transport in thylakoid membrane (Walker et al., 1999), an UDP-

glucose glucosyltransferase (GjUGT4) and a salt tolerance-like protein (Table 3.4). The third class comprised of nine DEGs encoding hypothetical proteins with no known functional annotation (Table 3.4). The fourth class consisted of one DEG (SuDEG1) that did not show any match to the nr sequences in the database (Table 3.4). The clones used in this study were isolated from up-regulated DEGs of Ho02-144.

3.3.4 Analysis of Expression of Differentially Expressed Genes Through Reverse Transcription Polymerase Chain Reaction

To validate the ACP-based expression data of the SuDEGs, semi-quantitative (sq)RT-PCR was performed using cDNAs from sugarcane of cold-stressed and unstressed leaves. Five SuDEGs encoding a cyclophilin (CyP; SuDEG2), a cell division protease (FTSH-5; SuDEG9), a cytochrome P450-related protein (CytP450; SuDEG26), a NADP dehydrogenase quinone 1 (NDH1; SuDEG27), and one hypothetical protein (SuDEG4) were analyzed (Table 3.5). In general, all five DEGs showed higher accumulation of their transcripts under cold stress than under unstressed control. However, all the genes showed an immediate upregulation after 24 h of cold stress in the tolerant clone Ho02-144 whereas their upregulation was observed at later time point (48 h or 72 h) of cold stress in the sensitive clone L79-1002 (Figure 3.4). The CyP (SuDEG2) mRNA accumulation was constitutive i.e., it was expressed under both control and stress conditions. It reached its maximum level of expression at 24 h after cold stress in the tolerant clone Ho02-144 and then showed downregulation during subsequent time points. In the sensitive clone L79-1002, a gradual upregulation of CyP was observed under stress although the level of expression was low in comparison to that in the tolerant clone at 24 h (Figure 3.4). FTSH-5 (SuDEG9) mRNA showed very high accumulation in Ho02-144 at 24 h and 72 h of cold stress. On the other hand, FTSH-5 showed a trend similar to SuDEG2; its expression was upregulated and reached its maximum at 72 h of cold stress. Similarly, SuDEG26 and SuDEG27 with similarity to cytochrome P450-related protein (CytP450) and NADP dehydrogenase quinone 1 (NDH1) respectively, were upregulated in both tolerant and sensitive clone under cold stress. SuDEG4 was very highly upregulated in both tolerant and sensitive clone under all time points of cold stress (Figure 3.4).

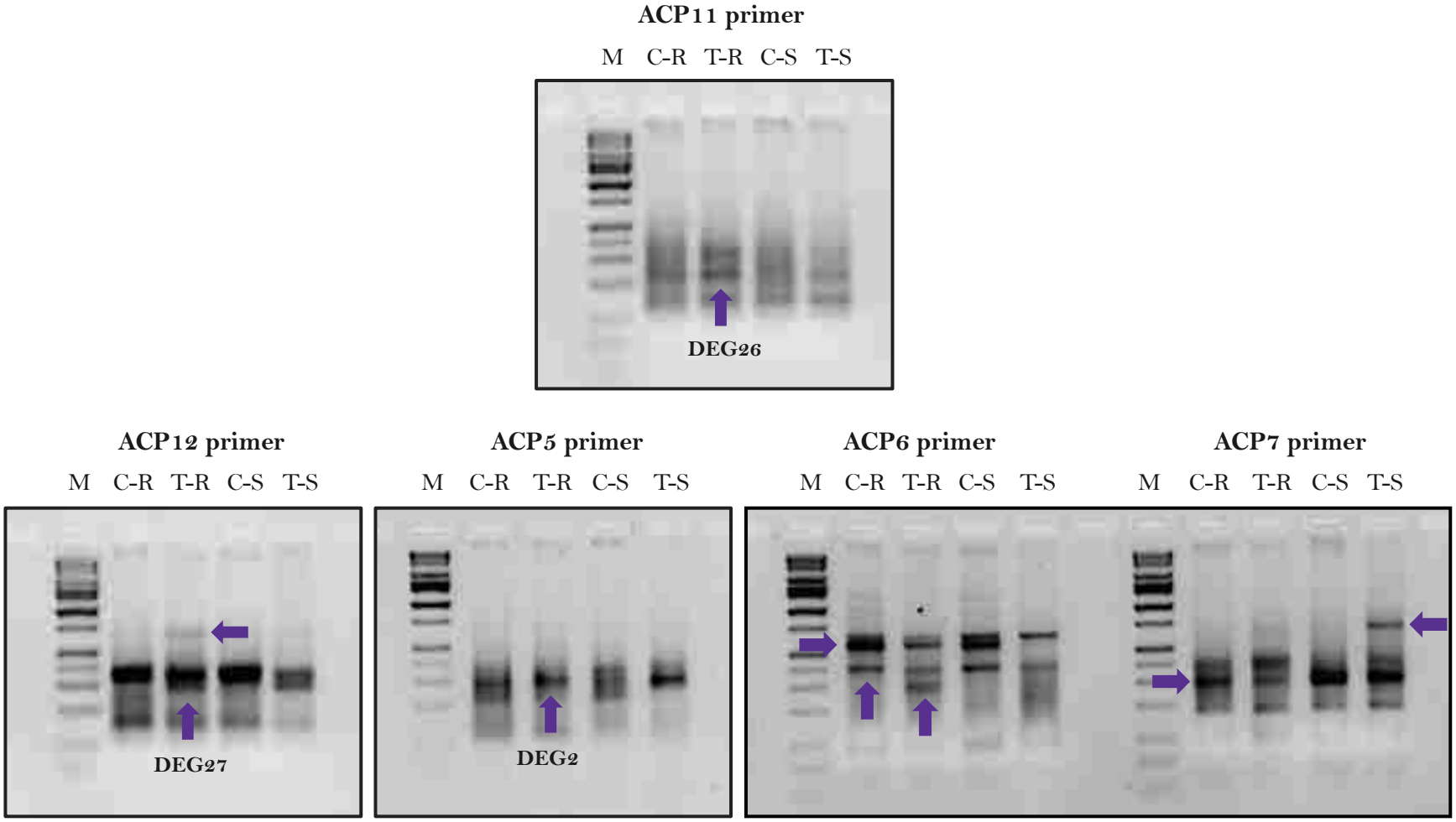


Figure 3.3. Identification of differentially expressed genes using gene fishing technique. Arrows show the upregulated genes after cold treatment. ACP: primers used, M: 1kb molecular marker, CR: control resistant, TR: treatment resistant, CS: control susceptible, TS: treatment susceptible.

Table 3.4. Similarity search results of sugarcane DEGs against NCBI nr database

Query (Clone)	Similarity (Gene or protein)	Blast Hit	Length (bp)	Ontology (GO)	Query coverage	Maxima identity	E-value
SuDEG2	Cyclophilin (CyP) (*Saccharum officinarum*)	GQ246462	166	Biological process, molecular function	83%	97%	2.00E-58
SuDEG22	Cyclophilin (CyP) (*Saccharum officinarum*)	GQ246462	173	Biological process, molecular function	78%	97%	2.00E-61
SuDEG3	14-3-3-like protein GF14-6 (*Zea mays*)	NM_001111880	149	Molecular function	53%	95%	9.00E-27
SuDEG5	Calcium transporter 1 (TRPV6) (*Sus scrofa*)	FJ268731	71	Biological process, Cellular component & molecular function	39%	100%	2.00E-04
SuDEG15	Calcium transporter 1 (TRPV6) (*Sus scrofa*)	FJ268731	140	Biological process, Cellular component & molecular function	23%	96%	4.00E-05
SuDEG8	Heat shock protein hsp23 (*Medicago sativa*)	JN103424	159	Biological process, molecular function	17%	100%	6.00E-04
SuDEG21	Heat shock protein hsp23 (*Medicago sativa*)	JN103424	135	Biological process, molecular function	51%	100%	2.00E-14
SuDEG25	Heat shock protein hsp23 (*Medicago sativa*)	JN103424	117	Biological process, molecular function	59%	100%	9.00E-16
SuDEG9	Cell division protease (FTSH-5) (*Arabidopsis thaliana*)	NP_568604	306	Molecular function	20%	96%	4.00E-03
SuDEG11	Mitochondrial heat shock protein 60 (*Tigriopus japonicus*)	EU306562	82	Cellular component & molecular function	34%	100%	2.00E-04

Table 3.4. (Cont.). Similarity search results of sugarcane DEGs against NCBI nr database

SuDEG12	Putative DEAD box ATP-dependent RNA helicase (ddx) (*Cancer pagurus*)	FR687022	36	Biological process & molecular function	83%	100%	5.00E-07
SuDEG16	Putative phenoloxidase activating factor (ppaf) (*Cancer pagurus*)	FR687023	42	Biological process & molecular function	88%	100%	5.00E-11
SuDEG23	Vitamin C Defective 2 (VTC2) (*Zea mays*)	NM_001156750	350		73%	89%	3.00E-74
SuDEG26	Cytochrome P450-related protein (CytP450) (*Arabidopsis thaliana*)	BM815951	138		50%	91%	3.00E-16
SuDEG27	NADP dehydrogenase quinone 1 (NDH1)	GH234512	296		62%	83%	3.00E-35
SuDEG3	Thylakoid assembly4 (*Zea mays*)	NM_001111472	118	Biological process & cellular component	48%	92%	2.00E-12
SuDEG6	UDP-glucose glucosyltransferase (GjUGT4) (*Gardenia jasminoides*)	AB555734	192	Molecular function	14%	100%	7.00E-04
SuDEG10	UDP-glucose glucosyltransferase (GjUGT4) (*Gardenia jasminoides*)	AB555734	100	Molecular function	28%	100%	3.00E-04
SuDEG20	Salt tolerance-like protein (*Zea mays*)	EU956191	270	Cellular component & molecular function	90%	86%	1.00E-57
SuDEG4	Clone SCUTLR1058C02 (*Saccharum officinarum*)	AY596553	350		92%	99%	9.00E-176
SuDEG7	Hypothetical protein (*Sorghum bicolor*)	XM_002452913	177		76%	91%	8.00E-43
SuDEG14	Hypothetical protein (*Sorghum bicolor*)	XM_002461393	165		46%	96%	4.00E-26
SuDEG17	Hypothetical protein (*Sorghum bicolor*)	XM_002466027	72		51%	100%	1.00E-10
SuDEG18	Hypothetical protein (*Sorghum bicolor*)	XM_002456344	226		83%	96%	4.00E-81
SuDEG19	Hypothetical protein (*Sorghum bicolor*)	XM_002456344	291		72%	95%	1.00E-85
SuDEG24	Hypothetical protein (*Sorghum bicolor*)	XM_002457541	258		52%	93%	2.00E-12
SuDEG28	Hypothetical protein (*Sorghum bicolor*)	XM_002455917	328		83%	88%	3.00E-85
SuDEG29	Hypothetical protein (*Sorghum bicolor*)	XM_002455364	123		76%	94%	2.00E-32
SuDEG1	No protein match	-					

Table 3.5. Primer sequences designed from selected DEGs of ACP-cDNA library for use in (semi)quantitative RT-PCR analysis

Clone ID	Similarity (Gene)	Left Primer (F)	Right Primer (R)	Annealing temperature (°C)	PCR product size (bp)
SuDEG2	Cyclophilin (CyP) (*Saccharum officinarum*)	CTCCACTACAAGGGCTCCAC	GACGGTGCAGACGAAGAACT	58	216
SuDEG4	Hypothetical protein SCUTLR1058C02 (*Saccharum officinarum*)	CGGAGGAACGACTATGAGGA	GTAACCGGGACAGGAGCATA	58	194
SuDEG9	Cell division protease (FTSH-5) (*Arabidopsis thaliana*)	CGAGTTGTTTGTCGCTTGAG	TACAAGCTGGGCTCTCCATT	58	125
SuDEG26	Cytochrome P450-related protein (CytP450) (*Arabidopsis thaliana*)	CACTACCCGCGGTACAAGAA	GTACTGGTCGGGCCAGAGG	58	150
SuDEG27	NADP dehydrogenase quinone 1 (NDH1)	TCATGAGCTTGTTCATTGACG	TTCCTACAAGCTGGGCTCTC	58	153
Reference gene	Elongation factor mRNA (*Saccharum officinarum*)	GAGAGGTCCACCAACCTTGA	GGGCTTGATGACACCAGTCT	61	180

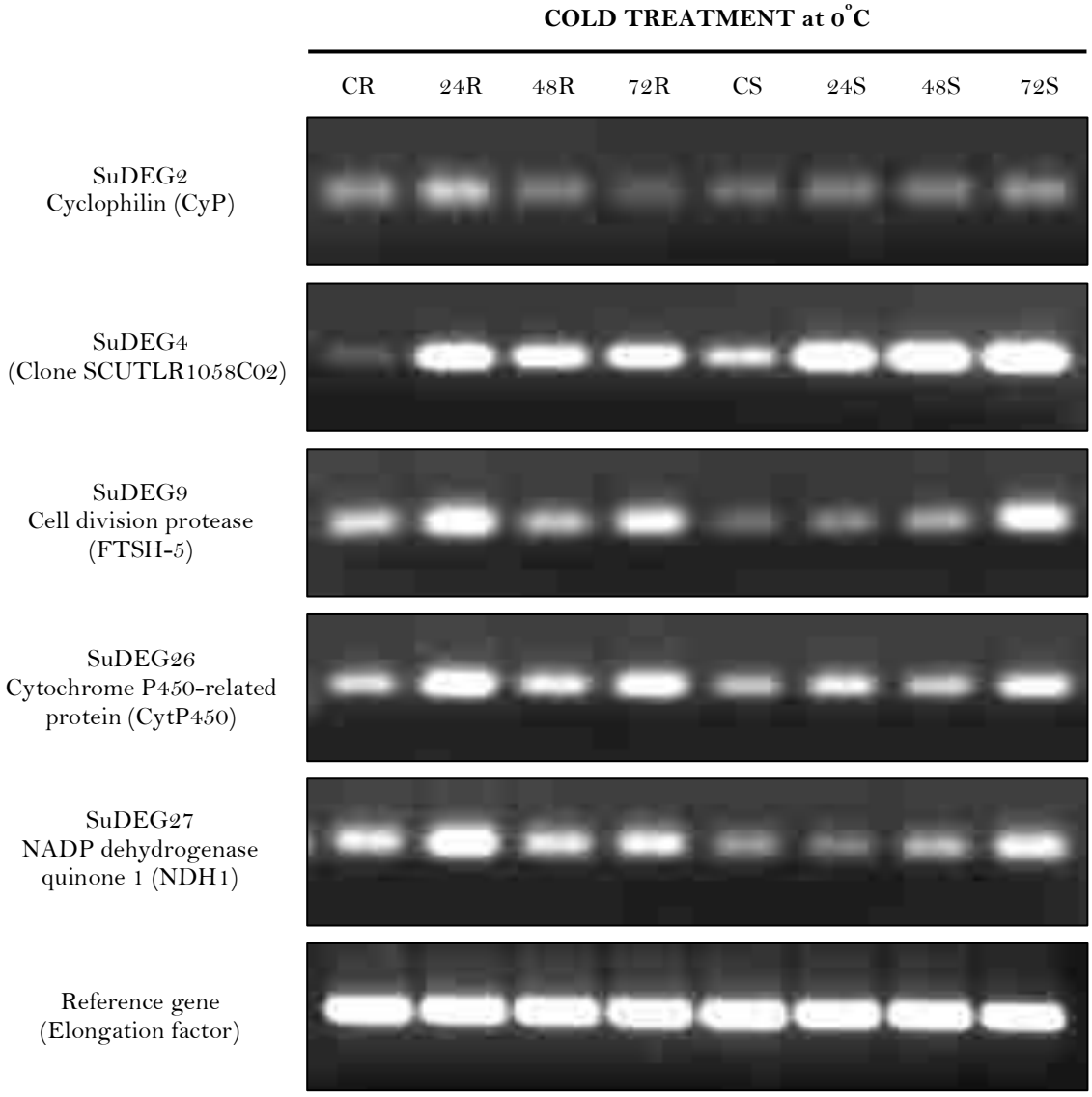
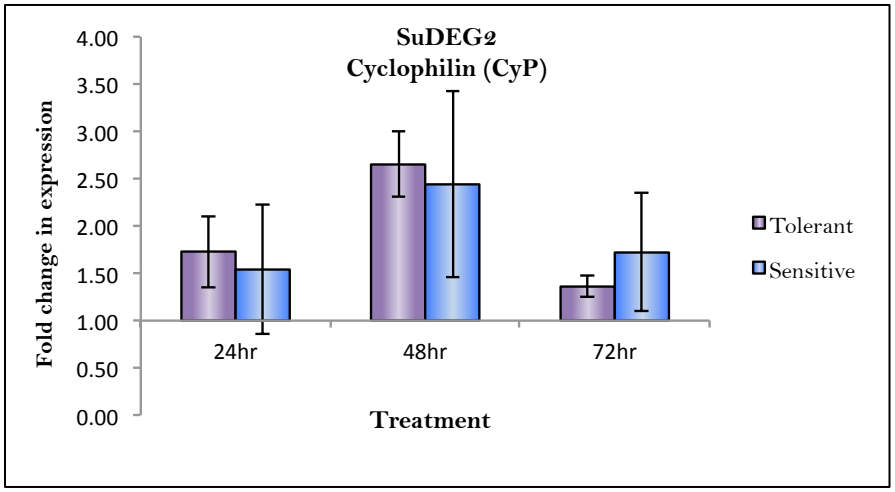


Figure 3.4. Semiquantitative reverse transcription (sqRT) PCR of five SuDEGs in sugarcane clones Ho02-144 and L79-1002. Sugarcane elongation factor was used as the internal control reference gene.

Quantitative (q)RT-PCR analysis was also performed with the cDNA, synthesized for sqRT-PCR, to validate the transcript levels of the five SuDEGs (Table 3.5) in the leaf tissue of sugarcane at various time points following cold treatment. The fold-change in gene expression in the cold stressed leaf relative to unstressed control leaf at different time point was normalized using the expression of the elongation factor (SoEF1α) gene from *S. officinarum* as control and is presented in figure 3.4. In general, the expression of the SuDEGs revealed distinctive gene expression profiles in the tolerant Ho02-144 and sensitive L79-1002 sugarcane clones. The relative expression level of the SuDEGs similar to CyP (SuDEG2), CytP450 (SuDEG26), and FTSH-5 (SuDEG9) was high under cold in both Ho02-144 and L79-1002 (Figure 3.5). The relative expression level of CytP450 was higher in the tolerant clone Ho02-144 than in the sensitive clone L79-1002 at all time points under cold. In the sensitive clone, the relative expression of all genes but CyP was highest after 72 h of cold treatment. CyP showed highest accumulation (2.44-fold) in the sensitive clone at 48 h after cold treatment. The expression of NDH1 (SuDEG27) and FTSH-5 was higher in the tolerant clone than the sensitive clone at 24 h and 48 h, but at 72 h of cold treatment their transcript accumulation was higher in the sensitive clone (3.72 and 9.80, respectively). The relative transcript abundance of CyP, NDH1 and FTSH-5 was increased by 1.72, 3.72, and 9.80 fold in the leaves of the sensitive cultivar 79-1002 and by 1.36, 2.83, and 6.61 fold in the leaves of the resistant cultivar Ho02-144 after 72 h of cold treatment. NDH1 showed 2.21-fold upregulation in Ho02-144 while it was down-regulated by 0.73-fold in L79-1002 after 24 h of cold stress. Figure 3.4 and 3.5 show a comparison of the gene expression profiling of SuDEGs obtained using sqRT-PCR (Figure 3.4) and qRT-PCR (Figure 3.5). The sqRT-PCR results, in most part, corroborated with the qRT-PCR results with a minor disagreement between the band intensities and fold induction. This is possible due to high sensitivity of fluorescence-based detection through real time PCR, which may not be precisely resolved through agarose gel based detection. The quantitative expression data of SuDEG4 did not agree with its semiquantitative expression, which could be due to the primer dimer formation during real time PCR that led to very low PCR efficiency. This gene could not be repeated due to the lack of sufficient cDNA.



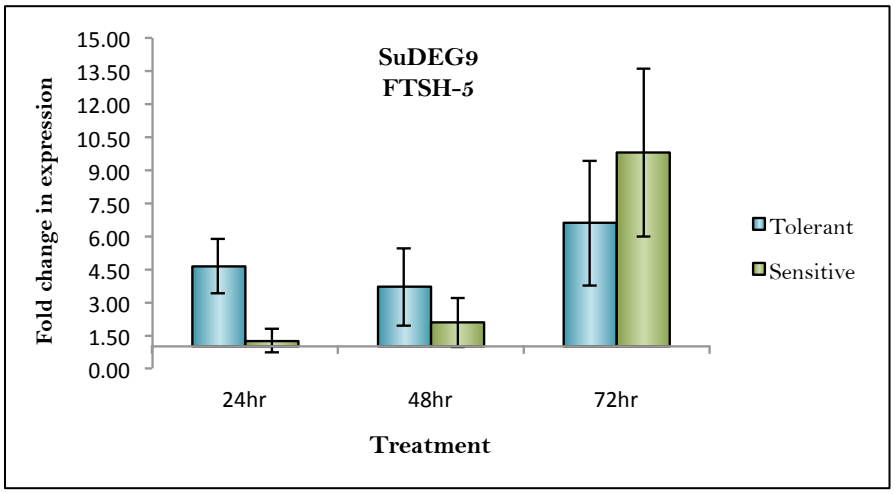
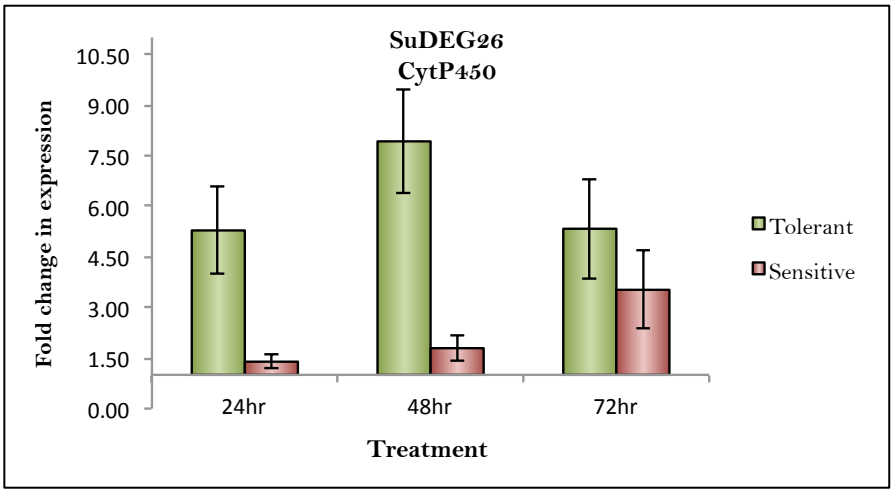


Figure 3.5. Quantitative reverse transcription (qRT) PCR of four SuDEGs in sugarcane clones Ho02-144 and L79-1002. Sugarcane elongation factor was used as the internal control reference gene.



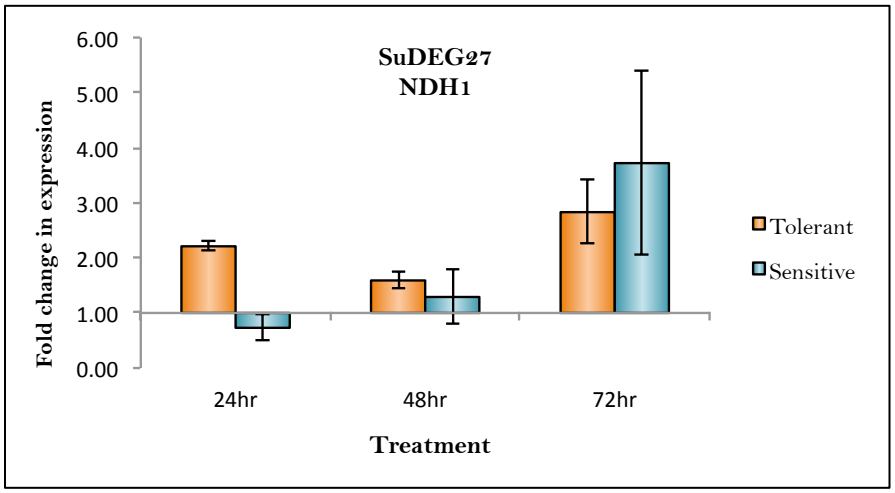


Figure 3.5. (Cont.). Quantitative reverse transcription (qRT) PCR of four SuDEGs in sugarcane clones Ho02-144 and L79-1002. Sugarcane elongation factor was used as the internal control reference gene.

3.3.5 SSR Mining of Cold Responsive ESTs

Out of one hundred and fifty cold-responsive ESTs of sugarcane that were mined for all perfect SSR motifs, twelve SSR containing ESTs were selected and used to design eSSR markers (Table 3.6). Similarity search resulted in only one out of 12 SSR-ESTs with no known function i.e., matching to "hypothetical protein". Gene ontology analysis assigned five sequences (NAC1, NAC23, glycine-rich RNA-binding protein 2, UDP-forming alpha-1,4-glucan-protein synthase, and CBL-interacting protein kinase 24) to both biological process and cellular component categories, while nuclear factor interleukin 3 regulated-like protein was assigned to both cellular component and molecular function categories. The remaining four (polyubiquitin 10, aquaporin PIP2-6, luminal-binding protein 3 precursor, and conserved peptide upstream open reading frame 5) were assigned to biological process (Table 3.6).

3.3.6 Estimation of Polymorphism by eSSR Markers in Sugarcane

Twelve eSSR primer pairs derived from cold-responsive ESTs were used to fingerprint a set of 48 commercial sugarcane hybrids, 14 *S. spontaneum* and two *S. officinarum* clones for studying their genetic diversity. Figure 3.6 shows representative gel images of the PCR profile obtained from these genotypes using eSSR primer 2, 3, 6, and 11. Eleven out of the 12 eSSR primers produced scorable bands among these genotypes, whereas one primer (eSSR8) failed to amplify. Due to the complex genome of sugarcane, most of the eSSRs produced more than two amplified products, which were considered to be dominant alleles. The eSSR primers amplified a total of 186 alleles with a range from one (eSSR4) to 71 (eSSR1), and 170 alleles were polymorphic (92 %; Table 3.7). The eSSR1 amplified the highest (69) number of polymorphic alleles while eSSR4 amplified only one allele. The polymorphism information content (PIC) of the eSSRs ranged from 0.11 (eSSR12) to 0.39 (eSSR4) with an average of 0.20 (Table 3.7). The mean PIC value of the eSSRs with dinucleotide motifs (0.23) and tetranucleotide motifs (0.21) were almost comparable, which was higher than the eSSRs with trinucleotides (0.14). In general, the eSSR primers, as expected, showed low level of polymorphism among the 64 genotypes.

Table 3.6. Primer sequences of 12 cold responsive eSSRs from the SUCEST database

Primer eSSR	Motif	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Expected product size (bp)	Expected EST homology	GI	Ontology
eSSR1	(TC)5	CGCCGATCCCAACAATCC	CTTGCCGGCGAAGATGAG	58	204	Polyubiquitin 10 protein	240255754	Cellular component
eSSR2	(GA)5	AATTCCCGGGCTACCATTAG	GTAGTGCATCACCAGCTCCTC	58	182	NAC23 protein	58013003	Biological process & Molecular function
eSSR4	(TG)5	GCAGCTATGTAGGGAGAGTCGT	CACCCAAGCTCAAGAAGAAGTG	60	192	Hypothetical protein 01g009520	242033121	Cellular component
eSSR7	(TC)10	ATTCAAATCGTCCCCTCCTC	GGTAGTAGTTCAGGCGTGTAAGG	60	185	Glycine-rich RNA-binding protein 2	195626496	Biological process & Molecular function
eSSR3	(CAC)5	GGGGCAGAGAGAGATCGAG	TCTCGAGGAAGTCGAGGTTG	60	169	Alpha-1,4-glucan-protein synthase [UDP-forming]	162463414	Biological process & Molecular function
eSSR5	(CGG)5	CGTCCGCTCTATTTCCCTTT	CCAGAAGAGTCCTAGAAGGAAGC	50	292	Nuclear factor interleukin 3 regulated-like protein	215398881	Cellular component & Molecular function
eSSR8	(GAG)5	ATCGATCCAGGAGCTTCCA	TCTTGGTCTCCTCCTTAGCAAC	58	163	Luminal-binding protein 3 precursor	162457903	Biological process
eSSR12	(GGT)6	GGTAGTGGTTCGGGCTAT	CCATATCCTTCACCTGACC	60	179	Glycine-rich_cell_wall_structural protein_2_precursor	149392269	Cellular component
eSSR6	(TTCT)6	AAGAACTCCACCTGCTGATCC	GGATGCTTCTCCTTCCTTCTTG	58	170	Conserved peptide upstream open reading frame 5	186495614	Cellular component
eSSR9	(AAGA)6	GTCTTTTTCGCCGATTCCAC	ACGTTCTTGGCGAACTTGAC	58	164	CBL-interacting protein kinase 24	116265964	Biological process & Molecular function
eSSR10	(ACCG)5	ATAGCCACCATCGACAGGAC	GTTGCTGGCGATGAATGAAC	60	154	Stress-induced transcription factor NAC1	88770831	Biological process & Molecular function
eSSR11	(CGGC)5	CTGTACCACCAGATCGTCCTC	CACTTGACCGAAGCTACTGCTA	58	194	Aquaporin PIP2-6	162459840	Cellular component

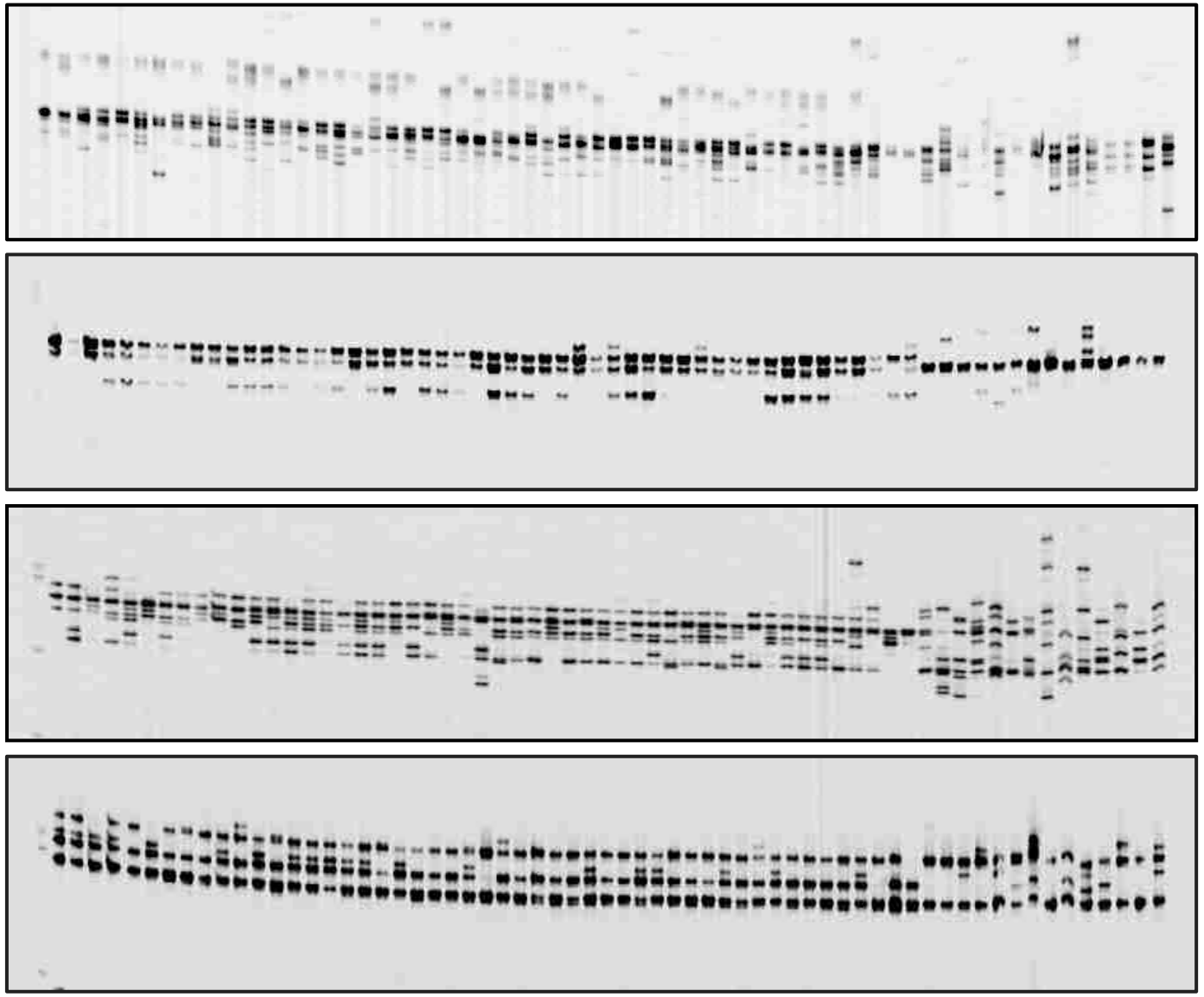


Figure 3.6. Genotyping of the 64 sugarcane genotypes (48 sugarcane clones and 16 ancestral parental clones) using eSSR primers 2, 3, 6, and 11.

Table 3.7. Polymorphism characterization of the 11 eSSR markers

Primer eSSR	Annealing temperature (°C)	No. of alleles	Mean no. of alleles	PIC	Mean PIC value
eSSR1	58	69	26.0	0.16	0.23
eSSR2	58	25		0.22	
eSSR4	60	1		0.39	
eSSR7	58	7		0.13	
eSSR3	60	6	7.0	0.14	0.14
eSSR5	58	10		0.18	
eSSR12	60	3		0.11	
eSSR6	60	22	12.3	0.17	0.21
eSSR9	58	5		0.23	
eSSR10	60	9		0.31	
eSSR11	58	13		0.13	
Average		**15.45**		**0.20**	

3.3.7 Genetic Similarity and Diversity of Sugarcane Genotypes Based on eSSR Markers

The average genetic similarity (GS) based on the Jaccard's coefficient with 170 polymorphic alleles among the 64 genotypes was 0.49, with the lowest pairwise genetic similarity value of 0.07 {between Spont57 (*S. spontaneum*) and hybrid clone 25, Spon57 and Offi50 (*S. officinarum*)} and the highest value of 0.74, between hybrid clones 34 and 42. High GS values were observed among commercial sugarcane clones.

Genetic diversity among the sugarcane genotypes were analyzed by both UPGMA cluster analysis and principal coordinate analysis (PCoA), based on 170 eSSR alleles (Figures 3.7 and 3.8). At 0.23 GS coefficient, the cluster analysis separated the 64 genotypes into two main clusters: Cluster I included the 48 sugarcane hybrids and two *S. officinarum* genotypes; and Cluster II comprised of the wild *S. spontaneum* genotypes (Figure 3.7). However, large genetic diversity existed between the cultivated clones and their ancestral parents. In general, the *S. spontaneum* clones were the most divergent in comparison to the commercial hybrids. For example, at 0.74 similarity coefficient, all the

sugarcane clones can be distinguished from each other except clone 34 and clone 42 whereas the spontaneum clones could be distinguished from each other at ~0.50 % GS. Principal coordinate analysis (PCoA) also supported the UPGMA cluster analysis where the polymorphic eSSR markers could clearly separate all the ancestral *S. spontaneum* clones from the cultivated sugarcane clones that also included the two clones of *S. officinarum* (Figure 3.8). The three principal coordinates explained 39.55 %, 5.21 % and 2.86 % of the total variation, respectively, with a cumulative variation of 47.62 %.

3.4 Discussion

Plants try to adapt and survive to a new environment by varying their gene expression pattern after sensing stress signals such as cold (Nogueira et al., 2003; Thomashow et al., 2010). The cellular adaptation to cold stress can be promoted by such alterations of gene expression (levels of mRNAs and proteins), which could play an important role in improving plant's tolerance to cold and other related stresses (Nogueira et al., 2003; Thomashow et al., 2010). The complexity of defense mechanisms has been suggested through molecular studies during the characterization of gene expression profile in *Arabidopsis*, blueberry and maize (Maruyama et al., 2004; Bell et al., 2008; Anderson et al., 1994; Nguyen et al., 2009) and is exemplified by the different cold-responsive genes that have been isolated from various plant species (Thomashow, 2001). Cold and oxidative-related stresses induce a set of common genes (Lee et al., 2009). Sugarcane, in general, is sensitive to cold temperature. This is attributed to the fact that selection has been exercised mainly on the sucrose trait. However, the existence of alleles from cold-hardy *S. spontaneum* in the sugarcane hybrids suggests there is a variation in their response to cold stress (Du et al. 1999). Earlier, cDNA subtraction-based expression profiling study in a Brazilian sugarcane clone showed expression changes in a number of genes in response to cold (Nogueira et al., 2003). In the present study an attempt was made to understand the cold-adaptation response of sugarcane plants through gene expression studies of two contrasting clones; Ho02-144 (cold tolerant) and L79-1002 (cold sensitive), and genetic variation among Louisiana sugarcane clones for eSSRs mined from cold responsive ESTs.

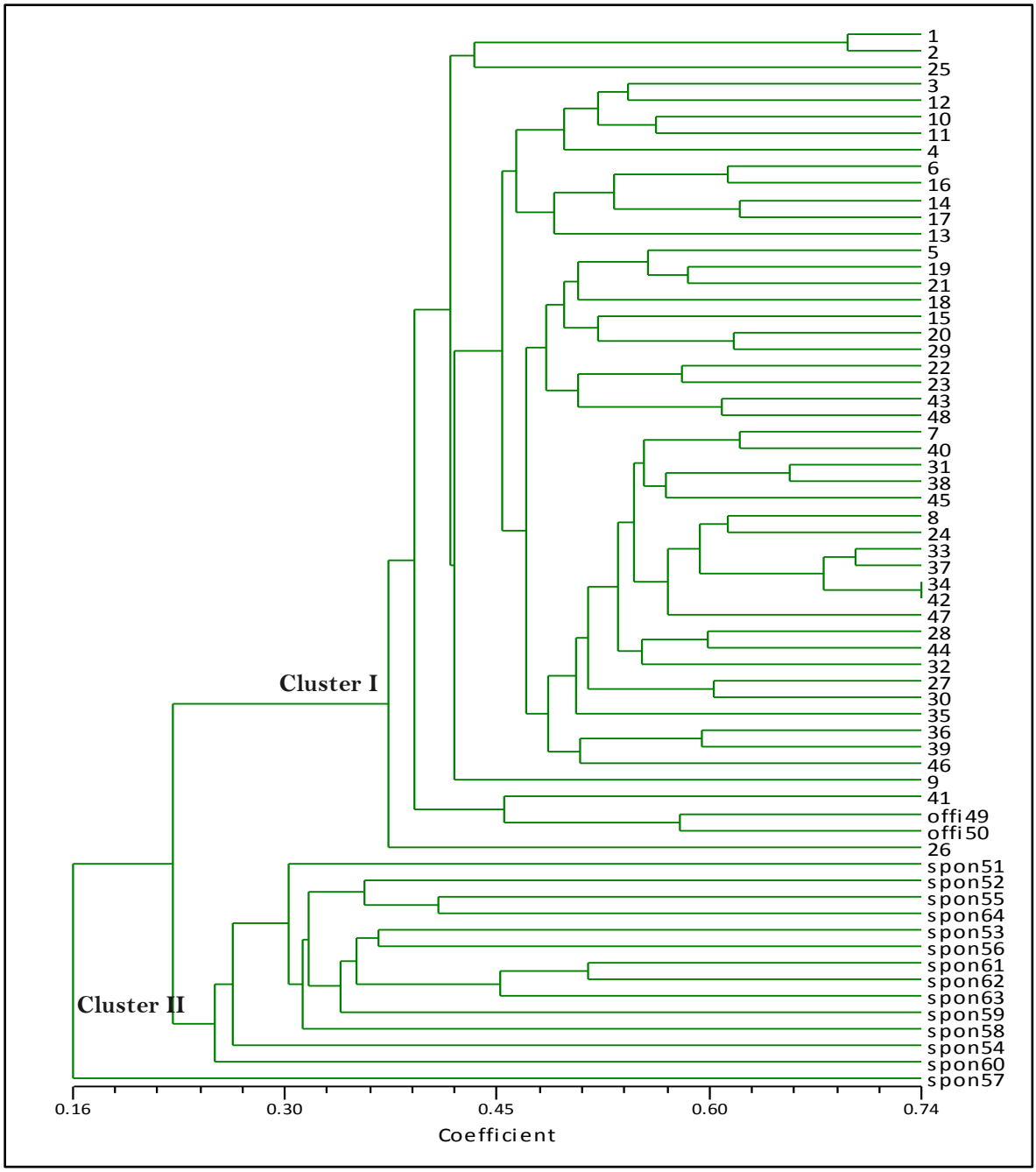


Figure 3.7. Dendrogram showing cluster pattern of 48 sugarcane clones and 16 ancestral parental clones, based on genetic similarity indices obtained from the eSSR markers.

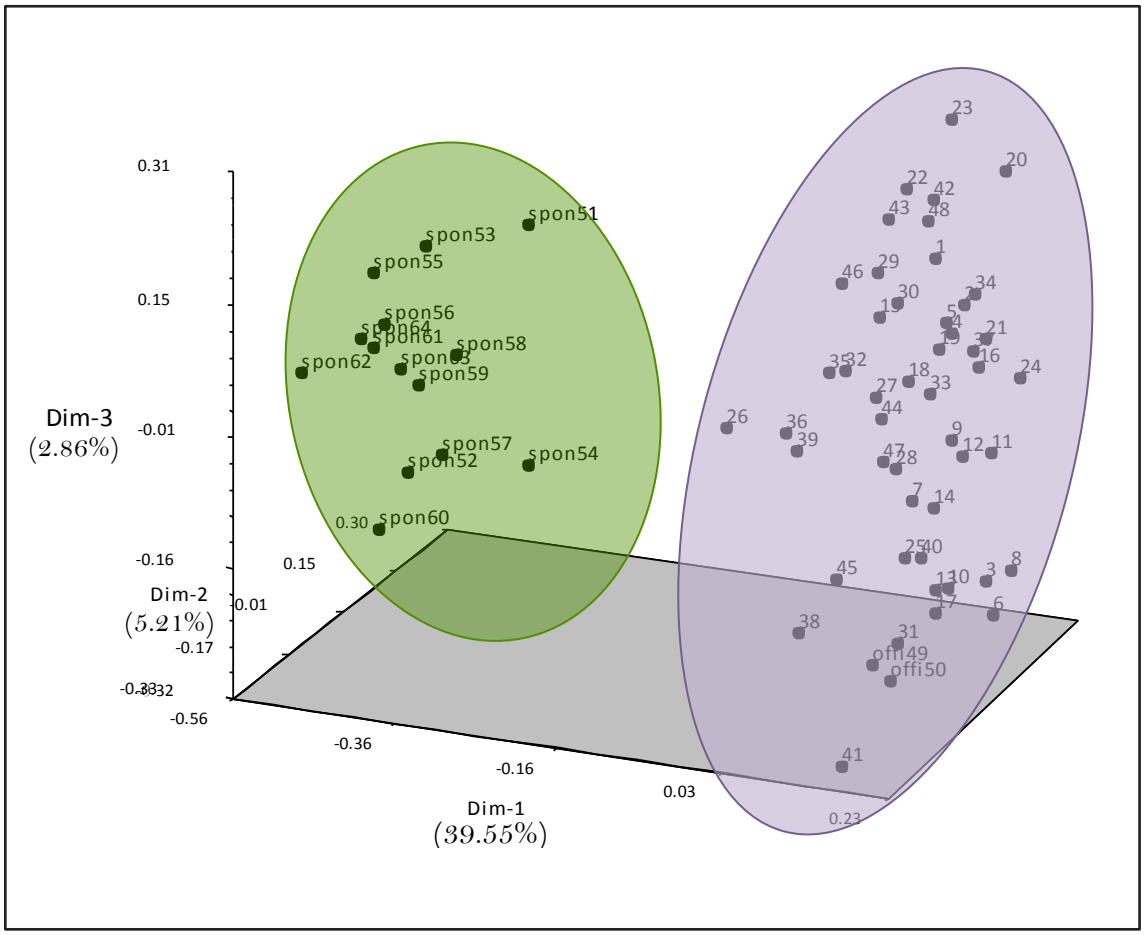


Figure 3.8. Three dimensional principal coordinate analysis (PCoA) plot showing distribution of 64 sugarcane genotypes based on eSSR markers.

3.4.1 Cold-responsive Genes in Sugarcane

A total of 40 SuDEGs (differentially expressed genes; 29 - upregulated, 11 - downregulated) were identified by the use of an annealing control primer (ACP) based gene fishing technique from Ho02-144 and L79-1002 when exposed to 0 °C up to 72 h. Eleven of these DEGs represented cold-induced genes identified in other plants.

SuDEG2 and SuDEG22, coding for cyclophilin (CyP) proteins (Table 3.4), are ubiquitous proteins that occur in diverse organisms and are involved in protein folding. CyPs play an essential role as molecular chaperones under various stresses (Marivet et al., 1994; Godoy et al., 2000). CyP gene was constitutively expressed at different time in sugarcane leaf by cold stress, but higher transcript accumulation was observed in the tolerant clone in comparison to the sensitive clone. This observation is consistent with that of Marivet et al. (1994) where higher amounts of CyPs in bean could facilitate the correct folding in the maturation processes of induced proteins under stress and were assumed to have defensive functions (Godoy et al., 2000). CyP2 was shown to be involved not only in the protein folding, but also in refolding of denatured proteins under cold stress in tobacco (Ruan et al., 2011).

Cell division proteases (FTSH) are also known to be induced under various stress conditions (Cui et al., 2005). In the present study, SuDEG9 was identified as a putative FTSH-5, which was significantly increased after 24 h of cold exposure. The enhanced expression of this protein in the tolerant clone under cold stress suggested that such proteins involved in the photosynthesis apparatus are implicated in protecting the photosystem from damage associated with cold stress (Liu et al., 2010). In the present study, the observation that the tolerant clone retained higher chlorophyll when compared to the sensitive clone could be explained by the protection and biogenesis of chloroplast due to overaccumulation of FTSH-5 gene in the tolerant clone (Jin et al., 2011).

The differential expression of SuDEG26 and SuDEG27 with protein identity to cytochrome P450-related protein (CytP450) and NADP dehydrogenase quinone 1 (NDH1), respectively suggested that genes involved in anthocyanin biosynthesis and cyclic electron flow around PSI are regulated under cold stress. It is possible that sugarcane NDH1 is induced in response to oxidative stress generated by cold stress (Mi et al., 1993).

Heat shock proteins are involved in the common physiological response of plants to temperature stress (Colinet et al., 2009). Heat shock proteins are molecular chaperones that are known to stabilize other proteins in response to low temperature (Bengyella and Pranab, 2011). In this study four ESTs (SuDEG8, SuDEG11; SuDEG21 and SuDEG25) showing similarity to heat shock proteins (Hsp23, mitochondrial Hsp60) were upregulated after cold treatment in the tolerant cultivar (Table 3.4).

Nakamura et al. (2004) observed that cold and salt stress induced accumulation of ATP-dependent RNA helicase (HVD1), a member of the DEAD box family, in barley. In sugarcane, the EST (SuDEG12) similar to HVD1 was induced after cold exposure in the tolerant cultivar. RNA helicase activity could play an important role as a regulator of CBF genes that are necessary for survival of plants under cold stress by using ATP hydrolysis as the source of energy (Nakamura et al., 2004).

The observations that tolerant and sensitive sugarcane clones responded differentially to cold stress at the level of mRNAs might suggest an operation of different signal cascades for activation of downstream cold-responsive genes and existence of superior alleles in the resistant clones.

3.4.2 Genetic Diversity Analysis

Microsatellite markers based on the expressed sequence tags (EST-SSR) have become markers of choice for the sugarcane scientific community not only for its ease of use, but also because of their inexpensive development and amenability to highthroughout operation (Da Silva et al., 2001). Because eSSRs are derived from the transcripts with a putative function, they are useful for functional diversity assay in natural populations or germplasm collections. The eSSRs can often be used as anchor markers for comparative mapping and evolutionary studies, and could prove useful for marker-assisted selection, especially when the markers reside in the genes responsible for a phenotypic trait (Varshney et al., 2005). Moreover, their cross transferability across different species could facilitate tracking of the ancestral *Saccharum* species-specific alleles in the commercial sugarcane hybrids. In this study, 12 cold responsive EST sequences harboring SSR motifs were used to design the eSSRs (Table 3.6).

Twelve eSSR markers were used to establish the genetic diversity among 64 genotypes that included 48 commercial sugarcane clones, 14 *S. spontaneum* and two *S. officinarum* clones. Ninety-two percent of the markers showed polymorphism among the sugarcane genotypes, which is higher than previous studies reported with the genomic and genic SSR markers of sugarcane (15 %, Pinto et al., 2006; 72 %, Aitken et al., 2005), The high level of polymorphism associated with the eSSRs in sugarcane genotypes could be due to high rate of replication slippage by large allelic diversity among and within the parental genotypes used to produce the commercial hybrids. *S. spontaneum* showed the most diversity, which further conformed to the finding of Suman et al. (2011). As compared with *S. spontaneum*, GS value was higher among sugarcane hybrids. These results corroborated earlier observations by Alwala et al. (2006) affirming that genetic diversity is, in general, low among cultivated sugarcane because of few parental clones involved in the development of the foundation clones through nobilization in breeding programs (Alwala et al., 2006). *S. officinarum* and *S. spontaneum* showed only 7 % similarity to each other, which reflected the distinctness of these two species.

The cluster analysis generated a dendrogram that showed a clear separation between cultivated sugarcane clones and ancestral parent clones (Figure 3.7). In sugarcane (Oliveira et al., 2009) and wheat (Chabane et al., 2007) the EST-derived microsatellites could distinguish the genotypes in accordance with their ancestral relationships. The grouping of the commercial hybrids with *S. officinarum* in Cluster I is expected because most of the modern cultivars have inherited the major part of the genome of *S. officinarum* during nobilization events (D'Hont et al., 1998). Similar results have been obtained earlier by the use of gene-based TRAP markers (Alwala et al. 2006; Suman et al., 2011). Thus genic markers seem to be useful to assess genetic diversity and discriminate between different species of sugarcane.

The average polymorphic information content (PIC) value of the eSSR primers was low (0.20). Low PIC (0.23) was also obtained in sugarcane by Cordeiro et al. (2001), which is due to the conserved sequences of the transcribed regions within the same genus (Chabane et al., 2007). Another reason for the low PIC level could be due to low frequency of transmission of the diverged sequence differences between *S. officinarum* versus *S. spontaneum* and cultivated sugarcane varieties (Berding and Roach, 1997). However, the present results demonstrated that eSSR markers were able to produce

adequate polymorphism for their use as molecular markers in sugarcane genetic diversity studies.

Chapter IV
Conclusions

- Molecular markers (RAPD and SSR) were found to be an appropriate means to assess genetic diversity in the smooth cordgrass accessions where the RAPD markers were more discriminative than the SSR markers.
- Similar levels of polymorphism were observed for both the marker systems with minor difference between dendrograms generated by each of the marker systems.
- Sufficient genetic diversity existed among the individuals to explore breeding interventions for genetic improvement of smooth cordgrass.
- Seventeen RAPD and 10 SSR primers amplified loci that were specific to 10 and five smooth cordgrass accessions, respectively. These unique genotype IDs of the smooth cordgrass accessions will be very useful for maintaining genetic identity and purity of the varieties.
- The results of this study suggested that multiple genes were induced in response to cold stress in sugarcane.
- Distinctive gene expression profiles were observed in the tolerant and sensitive sugarcane clone. Further studies will increase our understanding of the complex signaling cascade of the metabolic pathways that play key roles in cold stress tolerance of sugarcane.
- The polymorphic eSSR markers developed in this study demonstrated their usefulness to classify Louisiana sugarcane clones from their ancestral genotypes based on the distribution of cold-responsive alleles.

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Vita

Lina was born in Lima, Peru. In 2003, she completed her bachelor degree in biological sciences at the Universidad Nacional Mayor de San Marcos, Lima, Peru. Her professional experience as well as her passion into the world of plants began in 2004, when she joined to the International Potato Center in Lima to perform her undergraduate thesis under the supervision of Dr. Marc Ghislain. She completed her undergraduate thesis thanks to the scholarship obtained by the International Potato Center to conduct research on the characterization of the resistance to the late blight in potato. Based on her results, she received the title of Biologist with major in cellular biology and genetics in 2008. In 2009, she worked as a research assistant at National Institute of Agricultural Research in the department of molecular biology and plant genetic resource conservation for one year on modified genetically organisms in maize.

In 2010, Lina joined Louisiana State University in Baton Rouge, Louisiana, to pursue her graduate studies. During this time, she has had the opportunity to learn new molecular techniques, as well as, surviving to graduate school. Her thesis research involves utilization of molecular markers in assessing genetic diversity in smooth cordgrass and sugarcane. She recently has attended and presented a poster at the International Annual Meetings in San Antonio, Texas, and has been awarded in the category of poster competition. She is also an active member of Crop Science Society of America. She is currently a candidate for the degree of Master of Science in the area of agronomy and crop sciences, which will be awarded in May 2012.

Lina is an avid lover of shopping. She also enjoys traveling, food, photography and good company.