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AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS CHARACTERIZATION OF SEA OATS (*UNIOLA PANICULATA* L.) ACCESSIONS FROM SOUTHEASTERN ATLANTIC AND GULF COASTS OF THE UNITED STATES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Agronomy

by
Neil Prieto Parami
B.S., Mindanao State University at Naawan, Philippines, 1993
December 2003

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ABSTRACT

Uniola paniculata, commonly known as sea oats, is a C_4 perennial grass capable of stabilizing sand dunes. The genetic relationship and diversity among *U. paniculata* accessions from southeastern Atlantic and Gulf coasts of the United States was established by amplified fragment length polymorphism (AFLP) analysis. One hundred ninety *U. paniculata* plants belonging to nineteen different accessions were selected to represent eight locations; Texas, Louisiana, Mississippi, Alabama, Florida, South Carolina, North Carolina and Virginia. Twelve AFLP *Eco*RI+*Mse*I primer combinations generated a wide range of polymorphisms (42-81%) with a mean of 59%. *Eco*RI-CAG+*Mse*I-CGA, *Eco*RI-ACT+*Mse*I-CTC and *Eco*RI-CAG+*Mse*I-ACG have the highest polymorphic rate at 81%, 75%, and 72%, respectively. A total of 703 scorable bands were identified of which 417 were polymorphic. UPGMA dendrogram using NTSYSpc version 2.10t separated *U. paniculata* plants into three major groups with subclusters consistent to its collection sites. All the accessions from Texas (LA2, LA5, LA9 and LA17), Louisiana (LA15 and LA16), and Virginia (LA53) were orderly clustered in Group I together with two accessions from Florida (LA35 and LA39) indicating similar genetic profiles. Group II consisted of accessions from Mississippi (LA41 and LA47), Alabama (LA19 and LA21), and the other two accessions from Florida (LA29 and LA33). Group III comprised South Carolina (NC15 and NC19) and North Carolina (NC1 and NC11) accessions. Dice similarity coefficient shows a range of genetic similarity across all plants from 64-98%. Florida has the widest range of genetic variation among its genotypes followed by Mississippi. The principal component analysis (PCA) further confirmed the three major groups. Hierarchical analysis of molecular variance (AMOVA) after 1000 permutations showed highly significant results (*Fst* values, p=<0.001) for all the

sources of variation. The highest significant amount of genetic variation was observed at the state-level (47%) followed by among-genotypes (34%). Total variation among the accessions within a state was 19%. Calculated average molecular diversity over loci was highest in LA47 (0.07±0.04, Petit Bois Is., Mississippi), LA33 (0.07±0.04, Henderson Beach, Florida), LA15 (0.07±0.04, Fourchon Beach, Louisiana) and LA41 (0.07±0.04, West Ship Is., Mississippi). Overall, the populations of *U. paniculata* were genetically-diverse.

CHAPTER 1
INTRODUCTION

With increasing interest in dune restoration, some semi-tropical, perennial dune grasses have been extensively studied to find potential species that would help stabilize the diminishing sand dunes and eroding coastlines of nearly 141,915 kilometers (McBride et al., 1991) in the United States. Although coastal areas are highly admired for abundant natural resources, the dual pressure of rapid population growth and accompanying natural phenomena are known to cause much of the shoreline and coastal barriers tensions. Storms are damaging and substantially affect the varying segments of the coastal areas. Likewise, cold fronts generating southerly winds, waves, and heavy rainfall attributed to the relative erosion (Mendelssohn et al., 1991) of landmasses especially the dune systems in the coastal areas. A survey in the southeastern parts of the United States have reported approximately 1 to 50 meters per year of coastal retreat in the Gulf coast west of the Mississippi Delta to Texas (Walsh, 1994). Penland and Boyd (1981) noted that shoreline and erosion rates in the Louisiana coasts alone ranged from 5 to 50 meters per year. Bourne (2000) predicted that some of Louisiana's 3,460 kilometers of coastal wetland would be reverted to open water with a yearly decline of approximately 65 to 91 square kilometers. He further approximates that in the next 50 years, 1,800 to 4,500 square kilometers of landmass might vanish and an estimated public resource of more than $37 billion would be lost.

To address this gradual decline of the coastlines and the pressing need for sustainable management, many researchers have sought to utilize indigenous dune plant species for restoration and stabilization of the coastal zones. It is reasonable to assume the environment would replenish naturally in due time when favorable conditions prevail. Coastal areas are extremely dynamic environments and the need for immediate attention to prevent massive

land loss is as urgent as the circumstances often arises from direct conflict with natural coastal processes (McBride et al., 1991) like storms and hurricanes that occur annually.

Despite the overall decline in coastal resources, there is still some room left for cautious optimism. *Uniola paniculata*, commonly known as sea oats, is among the grass species that occupy the exposed foredunes environment in the coastal areas (Wagner, 1964) and is regarded as a pioneer species to tap for beach restoration endeavors (Johnson et.al, 1990). Having great sand-holding capacity (Westra et. al., 1966) along with other grasses like *Leptochloopsis virgata* and *L. condensata*, *U. paniculata* is a natural dune builder and has played a significant role as an integral part of the food web manifested by their co-existence with other species (Wagner, 1964). Yet, there are still some challenging areas to deal with in the ecology and biology of *U. paniculata*. As a target species for restoration efforts, a more detailed characterization of *U. paniculata* using the molecular marker technology is essential.

Characterization of flora and fauna has evolved rapidly and utilizes highly sensitive tools that allow a more accurate and comprehensive analysis far exceeding those obtained by morphological methods. Although there are some limitations, morphological markers or iso-enzymes were utilized to generate genetic maps of the species of interest for various purposes (Caetano-Anolles, 1997). The increasing demand and challenges to understand the complexity of nature and to meet the needs of humankind have brought the significant development of the DNA-based analyses (Karp and Edwards, 1997). The upsurge in DNA marker technology may have practical applications in the fields of agronomy, breeding, taxonomy, conservation, and ecology (Weising and Kahl, 1997). Obviously, the fundamental attraction of DNA markers is the immense amount of useful information that can be gathered. Given the countless and wider range of applications has become the most useful

tool to examine genetic diversity of organisms and generate genetic maps either for tagging traits of interest for germplasm conservation or enhancement (Caetano-Anolles, 1997).

In the context of plant breeding, the selection of superior cultivars of higher productivity is important. It can be achieved by identifying the quantitative traits loci (QTLs) detected by DNA markers, and thus providing the framework of linkage, and estimate of similarity and differences among important species for a breeding program (Stuber et al., 1999). Basing upon on the principle of marker-assisted selection, selection could be targeted to the molecular markers rather than for the traits itself (Karp and Edwards, 1997). These popular techniques which include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), and amplified fragment length polymorphism (AFLP) could be applicable and useful in characterizing genetic resources at molecular level to measure gene diversity and richness for ecological purposes.

1.1 Objectives

This study used the amplified fragment length polymorphisms system to examine the genetic diversity of *U. paniculata* naturally growing in the United States. This study is part of a general approach to rehabilitate the beach dune ecosystem through revegetation effort of sea oats along the coastlines of Texas, Louisiana, Mississippi, Alabama, Florida, South Carolina, North Carolina and Virginia. Considering the USACE (US Army Corps of Engineers) beach nourishment project specifications, the whole research program would examine the changing environmental condition affecting sea oat survival, reproduction and its life cycle. Thus, the long-term goal is to develop sea oat genotypes with superior

characteristics that would significantly fast track the re-stabilization of the eroding coastal zones.

With the use of amplified fragment length polymorphism (AFLP) technique, this study aims to characterize the population structure of *U. paniculata* naturally distributed along the southeastern Atlantic and Gulf coasts. Specifically, the objectives of this study are:

1) To establish the genetic structure of *U. paniculata* plants based on its similarity as resolved by the AFLP fingerprints;
2) To determine if significant genetic variation exists within and among the accessions of *U. paniculata* and how different are the accessions in different geographic regions as resolved by AFLP.

CHAPTER 2
LITERATURE REVIEW

2.1 Natural dune builders

Uniola paniculata is characterized as a perennial grass having deep fibrous root system capable of stabilizing sands as dune forms, and tolerates salt spray and saltwater reaching its habitat while thriving in high sand temperature (Wagner, 1964). Nutrient requirement is minimal thus, it is a good competitor withstanding sand abrasion and drifting (Dahl and Woodward, 1977). It has the capacity to advance seaward at a rapid rate by way of seedling dispersal under suitable condition as documented by Woodhouse et al. (1977) in his study on the invasion of *Uniola* in the *Ammophila* habitat. This often results in a jumble of dunelets that grow and rapidly merge to widen dune formation creating a new one infront of the old. According to Wagner (1964), Plucinet (1696), Catesby (1731), and Clayton (1739) were the first to describe *U. paniculata* but it was Linnaeus (1753) who formally named the American genus *Uniola*. Today, there are four species (*condensata, peruviana, pittieri* and *virgata*) known and distributed around the Pacific coast of Central America, Ecuador, Mexico, Bahamas Islands, and mostly in the southeastern United States as recorded in the World Grasses Database (www.rbgkew.org.uk/herbarium) using the DELTA (Description Language for Taxonomy) program version 5.11 (Dallwitz et al., 1995).

Morphology of *U. paniculata* as reported by several authors resembles (Wagner, 1964; Westra and Loomis, 1966; Harper and Seneca, 1974) to common agronomic oats. Sea oats is characterized having an average height of 125 cm with firm, flat and elongated lead blades, and similar to some common grasses, the roots are readily present at the nodes of its creeping rhizomes. The root characteristic lays the significant contribution of sea oats to dune formation as it collectively accumulates sands by its horizontal roots. Unlike the

American beachgrass, *U. paniculata* persist as a perennial cover after the sand has been stilled and it would significantly dominate in the pioneer zone (Walsh, 1994).

2.2 Reproductive system of *Uniola paniculata* L.

Reproduction of sea oats is demonstrated by its inflorescence characterized by its arching panicle composed of many laterally-compressed spikelets which are typically 2 to 2.5 cm long and 1 cm wide (Westra and Loomis, 1966). The spikelets are described to comprise variable number of florets (ranging from 5-20 florets), which are typically hermaphroditic, unisexual or sterile. Within each spikelet, the fertile florets are subtended by (3-6 sterile or neuter florets (Crewz, 1987). Each sterile hermaphroditic floret has the potential to mature a single caryopsis. The florets are composed of a large lemma and slightly smaller paleas nested together and between them are three stamens and an ovary (Wagner, 1964). Because of its herbaceous characteristic, buds are produced from the shoot's nodes. These buds are formed randomly around the circumference of the stem within the nodal region and sand deposition somehow stimulates elongation of the internodes providing more buds growth (Hester, 1985). Vegetative reproduction is then enhanced by its C_4 photosynthetic ability, *i.e.,* fixing carbon and allowing growth in low nutrient, low water, and high light environment (Zelitch, 1982; Robichaux and Pearcy, 1984). By means of asexual reproduction, a relatively large dune complex has the potential of being made up of a single plant or fragments from the plant (Wagner, 1964)

Harper and Seneca (1974) studied the flowering process of *U. paniculata* and indicated that normally three growing seasons are required for a seedling to reach maturity along the coast of North Carolina. Floral initiation is related to a temperature-dominated gradient. For example, southern populations have been observed to flower earlier than those

in North Carolina (Harper and Seneca, 1974). Walsh (1994) noted that sea oats is wind pollinated and cross-pollination may be required for sea oats to produce considerable number of seeds. Florets open and close in the early morning and only open once. Seeds are known to have high germination rates (Hester and Mendelssohn, 1987). In Louisiana, average seed numbers range from 0.00-9.53 per culm depending on the population (Walsh, 1994). Spikelets are rapidly disseminated by wind, and are usually buried by sand accretion. Ordinarily sea oats spikelets after dispersal, lie dormant until spring when all viable seeds germinate and apparently some dormancy mechanism prevents germination in fall even if the environment is favorable (Woodhouse and Hanes, 1966). Germination is prevented in the fall by the seed coat that, though permeable to liquids and gases, resists the expansion of the embryo referred to as thermally-sensitive physiological block (Wagner, 1964). By late May, all viable seeds germinate in few weeks and then greatest concentration of seedlings can found on the upper beach where sand accretion is light (Wagner, 1964).

Grisebach (1864) describes the grass having some flowers that are fertile and 3-androus while Hitchcock (1950) mentioned that the spikelets are apparently sterile, neither caryopses nor stamens found (Wagner, 1964). Although the spikelets of sea oats are deciduous, falling from the panicle over late fall and early winter, the florets remain attached to the rachis of the spikelet so that whatever seeds are contained are distributed as a unit (Westra and Loomis, 1966). Spikelets falling on sites of sand accretion are quickly buried and if this burial is not excessive (endosperm reserves allow the coleoptile to elongate no more than 6 inches) viable seeds will produce seedlings the following spring (Tyndall et al., 1986).

Seed production is rather low compared with its potential (Hester and Mendelssohn, 1990). Although fertile pollen is produced, about 70% of the ovules are aborted. *U. paniculata* is not a heavy seed producer (Westra and Loomis, 1966). The seeds remain in the spikelets and shatter from the panicles in late fall or winter and remain dormant until spring (Hester, 1985). Dormancy is considered to be due to a diffusible inhibitor that is retained by the testa even if the layer is readily permeable to water (Wagner, 1964). Moderate chilling of the moist seed during winter may be a factor in germination but it has been essentially without effect unless followed by temperatures in the range of 30-40^{o} C but indicated a good germination is possible without chilling if maximum temperatures are around 40° C (Westra and Loomis, 1966). These temperatures lead to the destruction of the germination inhibitors within the seed.

2.3 Breeding of *Uniola paniculata* L.

Crewz (1987) made good observations in his report on the reproductive response of *U. paniculata* in the natural population. He observed that *U. paniculata* is a plant having hermaphroditic flowering system thus it could be an obligate outcrosser and has a degree of self-compatibility, *i.e.,* pollen transfer would be probably among flowers of the same plant. He pointed out that Wagner's (1964) observation that sea oats did not show apomixes could be attributed of having sea oats samples that were not under proper environment induction. Under controlled condition experiment on sea oats where fruit set reduction was deduced to self-incompatibility, low fruit set could be due to the experimental set-up itself that inhibits pollination (Crewz, 1987).

As early as 1960, breeding of *U. paniculata* in nursery for transplantation to its natural habitat has been carried out (Woodhouse and Hanes, 1966). It was extensively

laborious and colonization of the transplanted plants was relatively slow. Although confronted with such limitation, nursery production of planting stock of sea oats was feasible. Resolving the germination issue of sea oats, Wagner (1964) studied the seed dormancy both on the dunes and in the greenhouse (Woodhouse and Hanes, 1966). Gibberellic acid treatment on seeds was effective in breaking seed dormancy (Woodhouse and Hanes, 1966). However, Westra and Loomis (1966) disagreed with gibberellic acid treatment instead he recommended thiourea with pre-chilling treatment by alternating high and low temperatures. Seneca (1972) reported his results on the germination and seedling response of three populations of sea oats distributed in Virginia and North Carolina region, Atlantic coast Florida, and the Gulf coast. He found out that seeds from Virginia and North Carolina required cold treatment while its seedlings showed homogenous vegetative potential. In the Atlantic coast Florida region, their seeds did not require cold treatment and seedling were found to have low potential for vegetative while the Gulf coast seeds showed response to cold treatment and the seedlings have the highest potential for vegetative growth (Seneca, 1972). In the succeeding years, Hester and Mendelssohn (1987) in a study involving Louisiana sea oats concluded that cold treatment did not enhance seed germination and was not required to break dormancy. It was noted, however, that room-temperature treatment yielded 88.8% germination without prior cold treatment suggesting that moist cold condition has done a great deal in reducing the time required to achieve 50% germination (Hester and Mendelssohn, 1987). Meanwhile, Bachman and Whitwell (1995) presented their results on the nursery production of sea oats utilizing preplant treatments of seeds. Thiourea was not effective in improving seed germination but gibberellic acid treatment at 100 ppm increased

and accelerated germination of freshly harvested seeds (Bachman and Whitwell, 1995). The authors concluded that poor seed germination could not be attributed to dormancy.

Subsequently, other researchers interested in *U. paniculata* studied the function of vesicular-arbuscular mycorrhizal (VAM) fungi on the survival and distribution of sea oats in the beach dune system. Association of these microorganisms with sea oats had been known to be beneficial to plant nutrition and sand grain aggregation (Sylvia, 1986). Colonization of root-associated fungi (VAM) is reported in sea oats and beach grass (*Ammophila* spp) and their presence is correlated to plant growth enhancement, translocation and transfer of nutrients to roots. In many inoculation experiments conducted by Sylvia and Will (1988) and Sylvia and Burks (1988) in both greenhouse and field settings, confirmed the significance of VAM fungi to dune grasses. Isolates of *Glomus spp, G. deserticola, G. globiferum, G. etunicatum* and *Paspalum notatum* were tested for its ability to colonize the roots, produce external hypae and, take up and transport P efficiently (Sylvia and Will, 1988). Sylvia (1986) documented the contribution of VAM network of extensive hypae that would also instigate substrate stabilization. The presence of asymbiotic N_2-fixing bacteria enhanced the plant growth due to the phytohormones produced by the microorganisms that affect nutrient uptake (Sylvia, 1986). Following this, in-vitro and greenhouse experiments were also done to determine whether the presence of bacteria affects spore germination and early hyphal growth in VAM fungi. Results have shown that spore germination was enhanced when bacteria and spores were physically contiguous on the filters in the soil suggesting involvement of non-volatile, diffusible substance, however no significant differences were found in plant-N or P increase due to the inoculation of bacteria (Will and Sylvia, 1990).

In 1986, micropropagation of *U. paniculata* was carried out by Hovanesian and Torres (1986) to determine if sea oat plants could be obtained through tissue culture. Their experiment produced large number of plants (384 plants) from a few collected caryopses and 95% of the caryopses used for callus induction germinated without specific treatment except for simple refrigeration. Shoots (75%) transferred to a root induction medium (1/2 MS + 15 g/l sucrose + 4.4 μM BA) initiated roots within 30 days and 95% had reached more than 50 cm in height.

2.4 Ecological and economic importance of *Uniola paniculata* L.

Wagner (1964) remarks that previous dune research had conspicuously missed the rather impressive body of research concerning individual components of the dune vegetation and their relationship to the whole. The pioneering works on dune ecology and its vegetation dated back in the year 1900 by Kearney (Ocracoke Island) and Harshberger (1900) in New Jersey, followed by Mohr (1901) in Mississippi, Lloyd and Tracy (1901) in Louisiana. Instead of analyzing the whole plant ecosystem in the coastal environment, Wagner (1964) studied the ecology of *U. paniculata* as one particular plant species within the dune-strand vegetation.

U. paniculata is known to be difficult to propagate and slow to establish so that it could not be planted alone. Nevertheless, its high sand trapping makes this grass species ecologically important. It is an excellent dune builder and could eventually dominate the foredunes when established and properly managed, thus many researchers have tried to resolve the limitation of *U. paniculata* (Woodhouse et al., 1977). Consequently, conserving and managing this grass species is equally important to help stabilize and reconstruct the eroded coasts' dune systems. In an experimental dune building and vegetative stabilization

study in Louisiana by Mendelssohn et al. (1991) transplanted *U. paniculata* with other grass species in dune building locations to help stabilize sand accumulation. Their observation was vegetation played a small role in sand accretion compared to fences' accumulated sand. However, in Gibson and Looney (1994) study, they presented an encouraging result on the colonization of *U. paniculata* among the other dune grasses on dredge spoil of Perdido Key, Florida. They reported the dominance of *U. paniculata* in a community with other species that continued to establish in new areas along the Key and *U. paniculata* populations were less likely to be affected by washovers than the other species. The high-density presence of *U. paniculata* in sand fence agreed with Mendelssohn et al. (1991) result that sand fences would increase survival rate of some transplanted species and concluded that the nature of vegetation on the dredge spoil indicates an ecologically equivalent habitat (Gibson and Looney, 1994). Nevertheless, Wagner (1964) recognized two major roles of *U. paniculata* as instrumental in the stability of dunes as it builds up, and as an integral part of the food web.

In terms of economic significance, *U. paniculata* has opened a window of opportunity for seedling nursery operations. Because of its importance to dune restoration projects, several breeders have established nurseries for commercial production and distribution. Protected by law in some states, *U. paniculata* has been regarded as precious coastal resource. Due to prevailing need in seedling supply, many nurseries are established to cope up with demands brought about by widespread transplantation activities.

2.5 Regulation protecting *Uniola paniculata* L.

Realizing the long-term importance of *U. paniculata*, several states around the United States have laws protecting this "endangered" species. Under the regulations of the Florida Department of Environmental Protection (FDEP) and other states like North Carolina, South

Carolina, and Georgia among others, sea oats is a protected grass species. According to FDEP guidelines, sea oats seeds cannot be collected, cut back or removed without a permit. Applying its regulation to all native dune vegetation, protection have extended against pedestrian traffic and unauthorized access to the habitat. Pruning can only be done as necessary and in strict compliance to FDEP guidelines while necessary replantation must be carried out to maintain as such delicate system.

2.6 Molecular markers systems

With much reliability and precision, molecular techniques have elucidated the complexity of life forms for varying fields of practical application. Many studies have shown how different molecular markers can be useful to analyze the existence of an organism, its genetic diversity and relationship within its habitat. Ultimately, even the most complex phenotype could now be simply understood through a series of discrete sets of information exhibited in a gene map providing abundant and adaptable reference points (Karp and Edwards, 1997). Regardless of the technique employed, results would fairly be successful in generating groupings of germplasm that appear to be agronomically and biologically meaningful (Hayes et al., 2000).

Either by hybridization or based on polymerase chain reaction (PCR), molecular markers are categorized into three basic techniques: (1) hybridization-based techniques or the non-PCR techniques, (2) arbitrarily-primed PCR and other PCR-based multi-loci profiling techniques, and (3) sequence targeted and single locus PCR. In forensic science, for example, these techniques have been used for criminal investigations with high degree of validity in defining clear anthropological and ancestral identity of individuals. Chakraborty (1997) relating to the extent of population structure in forensic databases generated by the

application of these techniques concluded that the largest component of genetic variation at all loci is due to inter-individual variation within the populations and the between-population variation is mostly due to racial classification of populations, making ethnic differences within any major racial group the smallest components of genetic diversity. Similarly, these molecular techniques are utilized in assessing botanical diversity by resolving genetic differences among individuals and population of plant species (Karp and Buiatti, 1996).

The arbitrarily-primed PCR and other PCR-based multi-loci profiling techniques do not need probe hybridization. These techniques commonly do not require sequence information from the genome but methodically differ in fragment separation, detection, primers length and sequence uses, and the stringency of PCR condition (Karp and Edward, 1997). Primers for these kinds of techniques use the multiple arbitrary amplicon profiling (MAAP), arbitrarily amplified DNA (AAD), random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR) and the DNA amplification fingerprinting (DAF) and typically initiate synthesis even when match with the template could not perfect. On the other hand, semi-arbitrary primers are used in selective restriction fragment amplification (SRFA) or the amplified fragment length polymorphism (AFLP). Vos et al. (1995) explained that the use of these primers is based upon the restriction enzyme sites that are interspersed in the genome. Protocol includes the restriction of DNA by the two restriction enzymes followed by ligation of the adaptors. PCR is carried out with generic primers that comprise a common part corresponding to the adaptors and restriction site, and a unique part that would correspond to selective bases. AP-PCR and RAPD are frequently used to detect polymorphism as taxonomic markers in population studies for a wide variety of organisms (Vogt et al., 1997).

2.7 Amplified Fragment Length Polymorphism (AFLP)

AFLP, in particular, has been proven a useful marker technology for characterizing genetic diversity in many agronomic crops (Vos et al., 1995). The AFLP has the unique property in that the AFLP markers can be used as both genetic and physical DNA markers (Vos and Kuiper, 1997). Specific advantages in this techniques include: (1) no prior sequence knowledge is required when applied to biodiversity studies, analysis of germplasm collections and genetic relationship; (2) AFLP markers can be scored codominantly which makes this technique more suitable for genetic mapping studies than in arbitrarily-primed PCR-based marker techniques; (3) AFLP is very useful in positional cloning because high marker densities can be obtained with modest efforts; (4) AFLP markers can be detected in almost any background or complexity allowing the use of AFLP to detect DNA markers both in genomic DNA and in clones of genomic DNA or pools of these clones (Vos and Kuiper, 1997). Eventually, AFLP would establish identities in determining parentage, in fingerprinting and distinguishing genotypes and varieties such as cultivars and clones (Weising and Kahl, 1997).

The feasibility of AFLP technique has been demonstrated in various field of interest. Ranamukhaarachi et al. (2000) applied a modified AFLP for rapid genetic characterization of plants. The study generates adequate resolution power with both self- and cross-pollinated plant species (*U. paniculata*, sea oats; *Pontederia cordata*, pickerel-weed; *Cynodon dactylon*, Bermuda grass; *Penstemon heterophyllus*) including cultivar, ecotypes and individuals within the population. Hayes et al. (2000) addressed genetic diversity, selection response and dissection of quantitative trait expression in barley (*Hordeum vulgare*) by utilizing AFLP. In sweetpotato, high polymorphism among the sweetpotato cultivars attained through AFLP

produced the grouping patterns confirming genetic diversity (Zhang et al., 1998). Partitioning between-region variability into pairwise distance gave a clearer a picture of the extent to which each region contributed to the total molecular diversity and determined as well the distribution pattern of diversity in different regions. Rapid assessment based on AFLP fingerprinting has been done in nectarine and peach varieties (*Prunus persica*) (Manubens et al., 1999) and in cassava (*Manihot esculenta*) (Sanchez et al., 1999). In cultivated alfalfa (*Medicago falcata*) and Kentucky bluegrass (*Poa pratensis*), mapping of AFLP and RAPD markers linked to apomeiosis and parthenogenesis (Barcaccia et al., 1998). The study dealt with the use of bulked segregant analysis (BSA) to detect RAPD and AFLP linkage groups in relation to unreduced eggs in a mutant of *M. falcata* and in the parthenogenesis in apomictic types of *P. pratensis*. In horticulture plants, Rajapakse et al. (2001) studied the AFLP marker development in rose for genetic mapping comparing three pairs of restriction enzyme. AFLP markers were used to identify and eliminate seedling produced through fertilization. With *Eco*RI/*Mse*I, primers with three selective bases for each restriction enzyme (+3/+3) have yielded an optimum number of bands better than the *Pst*I/*Mse*I.

The fluorescence version of AFLP referred to as fAFLP involve utilization of fragments on denaturing polyacrylamide gels either through autoradiographic or through fluorescence methodologies. Strictly following the method described by Vos and Kuiper (1997), fluorophore (fluorescent) labeled fragments generated in the selective PCR amplification would then be separated on a PAGE automated sequencer (Beere, 2001). Because PCR amplifications would be performed with high stringency, primers differing by only a single nucleotide base amplify a different subset of adapted fragments. By using combinations of primers with different selective nucleotides, a series of fAFLP

amplifications will sample loci from a large fraction of the genome and with the ability to control the number of selectively amplified fragments, an optimal number of fragments may be generated thereby avoiding complications associated with DNA smears or unacceptable levels of fragment comigration (Beere, 2001).

2.8 Genetic diversity

Genetic diversity can be defined as the sum of genetic information contained in the genes of individual plants, animals and microorganisms. Thus, every individual is a storehouse of an immense amount of unique information in the form of traits, characteristics, varying behavior and interactions (Tanskley et al., 1989). It is estimated that the number of genes ranges from about 1000 in bacteria to more than 40,000 in many flowering plants (Vos and Kuiper, 1997). While each species consists of many organisms, it is common knowledge that virtually no two members of the same species are genetically identical. Therefore, biologists have argued that this value of diversity is likely to be associated with the variety of different genes that can be expressed by organisms as potentially useful phenotypic traits or characters such as morphological features and functional behavior (Tanskley et al., 1989). Because we do not know yet precisely which genes or characters will be of value in the future, we must treat all as having of equal value and then the greatest value for conservation will come from ensuring the persistence of as many different genes or characters as possible (Karp and Edwards, 1997).

Genetic diversity, selection response, and dissection of quantitative trait expression are typically issues of importance and interest to all plant breeders and ecologists. Knowledge of genetic diversity and relationship among genotypes is useful to understand germplasm organization for more efficient selection of better cultivars (Smith, 1997) while

for effective conservation relating to ecological application, diversity information would help in defining appropriate geographical scales for monitoring and management by understanding gene flow mechanisms, origin of individuals, and the role of its existence in a specific zone in a common habitat (Rademaker and de Bruijn, 1997).

One of the more broadly shared and economically defensible values for conserving and analyzing diversity may be seen to lie in ensuring continued possibilities both for adaptation and future use. Fingerprinting, or genetic marking, provides abundance and the huge amount of information derived from DNA is ideal for population studies and species identification (Morgante and Olivieri, 1993). The characterization of marker profiles would further provide direct, reliable, and efficient strategies in assessing genetic variation of germplasm (Mohan et al., 1997). Most likely, the success in any genetic conservation effort and breeding program would rely on understanding the amount and distribution of genetic diversity present in the gene pool; and for conservation to be effective and sustainable, utilization of the information on genetic diversity is needed to define appropriate measures in geographical scales for management (Schierwater et al., 1997). Diversity questions must then be answered from the species level up to the population and within population levels.

For conservation, systematic, ecological, and evolutionary studies, identification of taxonomic units and uniqueness of species is essential (Rademaker and de Bruijn, 1997). They identified three families of repetitive sequences including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence and the 154 bp BOX element, which permit differentiation at species, subspecies and strain level. The results provide information defining the distinctiveness of species and their phylogenetic position (Karp and Edwards, 1998).

Schiewater et al. (1997), on the other hand, illustrated the principal potential of AAD markers in analyzing genealogical relationship at the intraspecific and interspecific level to identify diagnostic markers for systematic units providing cladistic characters for inferring phylogenetic relationship in sea turtles. Whether the loss of individuals from one site is compensated with the migration of turtles from other nests was resolved the problem by generating molecular genetic data. Significantly, data gathered from the above studies would help determine the origin of individuals; how species are distributed; how genetically distinct each species from other individuals in a given population; and how much variation is present, thus a proper management scheme could be effectively established (Karp and Edwards, 1997).

Likewise, DNA markers have proven its efficiency in characterizing artificial or cultivated accessions, collection, germplasm and breeding lines (Smith, 1997). Accordingly, diversity analysis at this level would resolve the function of different genetic classes, its similarities and their evolutionary relationship with wild relatives as the important source of genes with qualitative traits (Dweikat and MacKenzie, 1997; Rafalski, 1997; Vogel and Scolnik, 1997; Vogt et al., 1997; Vos and Kuiper, 1997; Weising and Kahl, 1997). Furthermore, it permits the organization of germplasm of elite lines for more effective parental selection because marker assisted selection would facilitate the use of exotic germplasm in breeding thereby making diversity within the collection more structured and accessible (Karp and Edwards, 1997). Chakraborty (1997) stressed that it also important in establishing cultivar identity, breed or clonal identification, and on how genetic variation is distributed within populations.

Marker systems have been an attractive choice because of the relative speed with which genetic information can be gathered. Measures of diversity are needed to determine the 'where' of *in situ* conservation action rather than the 'how', particularly in deciding which combinations of available areas could represent and help sustain the most diversity value for the future (Schierwater et al., 1997) and should quantify a value that is both broadly shared among the people for whom they are acting and considered as being in need of protection (Smith, 1997). This is because genetic erosion of several crops has already occurred leading to the world's dependence for food on just a few species. Currently, a mere 100 species account for 90% of the supply of food crops and three crops – rice, maize, and wheat – account for 69% of the calories and 56% of the proteins that people derived from plants (Hayes et al., 2000). With the longer-term possibility of empirically testing the predictive utility of economically important mapped genes, such molecular approach in germplasm characterization would be effective in providing plant breeders an incentive in sustainable genetic resources utilization for conservation (Karp and Edwards, 1997).

2.9 Population structure

In the area of inferring population structure, a subjective approach is a sensible way to incorporate diverse types of information. The definition of population is typically subjective based on linguistic, cultural or physical characters as well as the geographical location of the sampled individual (Pritchard et al., 2000). Genetic variability is expressed in individual organism creating complex structuring of its population as a function of its ability to adapt to constant pressure within its environment (Hey, 1997) and for very closely related populations, it is assumed that allele frequencies are correlated across populations (Pritchard et al., 2000). Within species, genetic exchange has been traditionally emphasized as the

determinant of genetic structure (Sales et al., 2001), and historical events such as range expansion, range fragmentation and population bottlenecks would be strong determinants of population genetic structure (Schaal et al., 1998). Shared common ancestry and similar selective regimes could also account for genetic cohesion, thus in many groups genetic exchange across species range is restricted either by wide geographical distribution of populations or by limited pollen and seed dispersal (Schaal et al., 1998).

As explained by Schaal et al. (1998) and Slatkin (1987), the genetic structure of plant populations reflects the interactions of different processes, including the long-term evolutionary history of the species, its shifts in distribution, habitat fragmentation, population isolation, mutation, genetic drift, mating system, gene flow, and selection. Using both quantitative and molecular technique, the authors have accurately resolved the population structure of endemic plant *Digitalis minor*. The reliability of their results has provided a basis for the *in situ* conservation and exploitation of this plant genetic resource. Thus, an understanding of the extent and distribution of genetic variation is essential for devising sampling strategies, which would efficiently capture genetic diversity for selection trials and the subsequent use of materials that fulfill the dual aim of high genetic variation and reasonable performance (Sales et al., 2001).

The complexity of the genetic species as described by Hey (1997) could be attributed to the concept of genetic drift within the scenarios of the population structure. Templeton (1989) suggests that a boundary exists within a given environment characterizing a defined structure of a group of organisms where the level of gene flow or demographic exchangeability is high relative to the level with organisms outside of the group. In a random mating population for instance, where mating of other organisms outside the group does not

occur, genetic drift must be creating a kind of individual with defined structure and boundaries in space and time (Hull, 1976). In the case of isolation by distance (Wright, 1943), however in which the times of possible co-ancestry for a given pair of DNAs are proportional to the physical distance between the members of that pair, the pattern of genetic variation among organisms may not be structured but may follow a continuous pattern over some environmental landscape (Hey, 1997).

Another kind of population structure, may lead to nested levels of demographic exchangeability or gene flow with multiple nested boundaries to the pattern of genetic drift just like in the case of *Escherichia coli* where genetic drift may occur over a short time scale among the cells in a single colony on a petri dish and over a longer time scale among the population of cells within the intestine of a single mammal (Hey, 1997). Thus, it suggests hierarchy of levels of genetic drift and where individuality occurs at multiple nested levels (Hartl and Dykhuizen, 1984). Meanwhile, another pattern not clearly consistent with shared genetic drift would arise when two sexual populations share genetic drift for just a portion of the genome, *i.e.,* under natural selection, they may share drift over the entire genome with the exception of a single region, though these populations may still generate hybrids with some reproductive success and share drift at parts of the genome that are not linked to those that are under differential natural selection (Hey, 1997).

Dobzhansky (1950) envisioned the existence of a boundary, a partition in the magnitude of gene flow such that there was a point when species could be defined. He defined the smallest Mendelian population as a panmictic unit, and envisioned larger Mendelian populations to be groups of panmictic units that engaged in gene flow. However, the concept of a Mendelian population does not by itself imply the existence of such a

boundary because the causes of genetic species, both mutational and environmental, would somehow create groups of organisms with periods of uniform genetic drift in which the probability of recent co-ancestry for any pair of DNAs has little variation (Hey, 1997). Rapid genetic drift may result either from ecological circumstances that sharply curtail reproduction or from the appearance of a strongly favored mutation as in the cases that occur among plants, especially those that rarely outcross or have limited gamete dispersal and experience isolation by distance (Levin, 1979). During reproduction, rapid genetic drift can occur for a tightly linked portion of the genome as a result of advantageous mutations or an abundance of deleterious mutations likewise, environmental changes or mutations may create genetic species from groups of organisms (Hey, 1997).

2.10 Scoring, data analysis and statistical softwares

In sweetpotato, the different fragments produced with each primer were treated as a unit character and numbered sequentially for scoring and analysis (Zhang et al., 1998). They scored genotypes for the presence (1) and absence (0) of each fragment while only those fragments with medium or high intensity were taken into account and the monomorphic fragments. A matrix of pairwise distance defined by Excoffier et al. (1992) and a matrix of similarity based on simple matching coefficients by Sneath and Sokal (1973) were calculated using the ARLEQUIN version 2.0. A dendrogram was then generated to determine the similarity-based relationship between cultivars following the unweighted pair group model with arithmetic mean algorithm (UPMGA) using SAHN clustering analysis in NTSYS-pc version 2.10t (Rohlf, 1992). A nonparametic permutational procedure was followed to test and extract variation of individual within and between regions. Variation between regions

was then partitioned into pairwise distance regions to examine regional contribution to total molecular diversity (Zhang et al., 1998).

Sales et al. (2001) in their study on the population genetics of endemic plant species *Digitalis minor* have presented comprehensive statistical analyses of their data. Types of observation like the total scorable bands; those bands with frequency in each population less than 1-[3/N] where N is the number of sampled plants in the population (Lynch and Milligan, 1994); and those bands that fulfilled Lynch and Milligan (1994) criterion within each island were used for the analyses. Mantel test in NTSYS-pc was conducted for complete correlation analysis. Dendrograms were produced by neighbor-joining (NJ) cluster analysis available in NEIGHBOR from the PHYLIP 3.5c software package (Felsentein, 1993). Then the NEIGHBOR and CONSENSE program in PHYLIP were applied to generate 100 trees to be used to produce consensus tree. DCENTER and EIGEN in NTSYS were also conducted using the Apostol distance matrices to perform principal coordinate analysis (PCO). TREEVIEW (Page, 1996) was used to generate and print all their dendrogram results. In analyzing the genetic structure of *D. minor* populations, the authors performed AMOVA available in the WINAMOVA 1.5 program. AMOVA approach in ARLEQUIN version 2.0 was done to calculate overall, individual-locus and pairwise estimates of the correlation of alleles within subpopulation (*Fst*) for both the origin-based and model-based groupings. Their analyses were carried on using *F* statistics (*Fst*) as a secondary approach to estimate population genetics. Using RADPFST from RAPDPLOT, *Fst* was estimated from the RAPD bands that fulfilled the Lynch and Milligan (1994) assumption.

CHAPTER 3
MATERIALS AND METHODS

3.1 Plant profile

The taxonomic classification of *Uniola paniculata* L. as provide by PLANT Database of USDA-NRCS (http://plants.usda.gov) is given below (Table 3.1). Likewise, a summary of its morphology, physiology, reproduction and growth requirement is presented (Table 3.2).

Table 3.1 *Uniola paniculata* L. as classified by USDA-NRCS PLANT Database (2002).

Kingdom	Plantae
Subkingdom	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)
Division	Magnoliophyta (flowering plants)
Class	Liliopsida (monocotyledons)
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae (grass family)
Genus	*Uniola*
Species	*paniculata*

Watson and Dallwitz (1992) reported the genus *Uniola* L. as a tetraploid with a chromosome number of 2n=40 and a base chromosome number of x=10.

Importantly, *U. paniculata* shall not be confused with *Chasmanthium latifolium* (known as the northern sea oats and looks very similar) because this grass species thrives in rich woods and cannot be planted in beach dunes (USDA NRCS, 2002).

3.2. *Uniola paniculata* L. distribution and source sites

Populations of *U. paniculata* are documented to naturally thrive in Region 1 (Northeast), Region 2 (Southeast) and Region 6 (South Plains) of continental United States. Under the wetland indicator status, *U. paniculata* is classified as facultative upland type

Table 3.2 Summary of *U. paniculata* L. characteristics as detailed by USDA-NRCS PLANT Database (2002).

A. Morphology and physiology			
Active growth period	*Spring and Summer*	Fruit/Seed color	*Brown*
After harvest regrowth rate	*Slow*	Fruit/Seed conspicuous	*Yes*
Growth form	*Rhizomatous*	Growth rate	*Slow*
Height, Mature (feet)	*6*	Shape and orientation	*Erect*
C:N ratio	*High*	Nitrogen fixation	*None*
Fire resistant	*No*	Toxicity	*None*
B. Growth requirement			
Adapted to coarse textured soils	*Yes*	Moisture use	*Low*
Adapted to medium textured soils	*Yes*	pH	*6-7.50*
Anaerobic tolerance	*Medium*	Planting density per acre	*4,480-19,000*
$CaCO_3$ tolerance	*Medium*	Precipitation	*35-65*
Drought tolerance	*High*	Root depth minimum (inches)	*20*
Fertility requirement	*Medium*	Temperature, minimum (°F)	*7*
Salinity tolerance	*Medium*	Shade tolerance	*Intolerant*
C. Reproduction			
Bloom period	*Early Summer*	Propagated by sprigs	*Yes*
Fruit/Seed abundance	*Low*	Seed per pound	*4500*
Fruit/Seed period begin	*Summer*	Seed spread rate	*Slow*
Fruit/Seed period end	*Fall*	Seedling vigor	*Low*
Fruit/Seed persistence	*Yes*	Vegetative spread rate	*Moderate*

indicator, *i.e.,* it usually occurs in non-wetlands (estimated probability 67-99%), but occasionally found on seasonally and semi-permanently flooded wetlands (estimated probability 1-33%) (US Fish and Wildlife Service, 1988).

For this study, seven states in the southeastern Pacific and Gulf coasts were selected as source sites of *U. paniculata*. Source sites include the states of Texas, Louisiana, Mississippi, Alabama, Florida, South Carolina, North Carolina and Virginia (Figure 3.1).

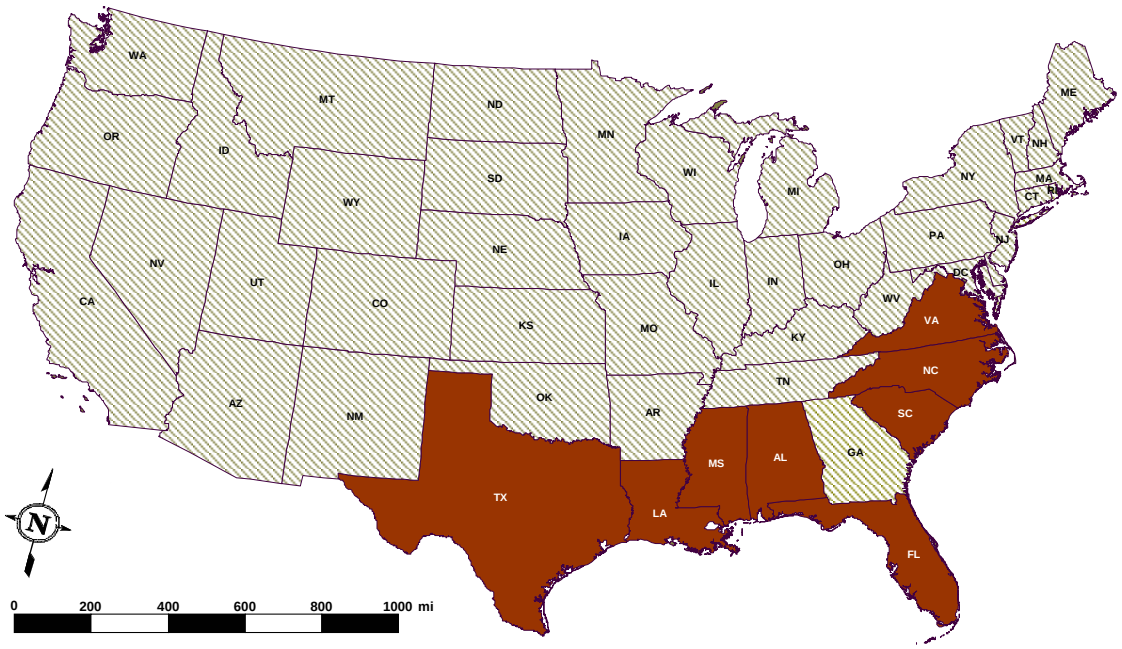


Figure 3.1 Map of continental United States showing collection sites [Region 1 (Northeast) = Virginia; Region 2 (Southeast) = North Carolina, South Carolina, Florida, Alabama, Mississippi, Louisiana; Region 6 (South Plain) = Texas] of *Uniola paniculata* L. seed materials and its natural distribution along the Atlantic and Gulf coasts.

Spikelets from the natural population of *U. paniculata* were collected as the primary source of plant materials. *U. paniculata* panicles were harvested during September to December 2001 from the seven states: Texas, Louisiana, Mississippi, Alabama, Florida, South Carolina, North Carolina and Virginia, as indicated in Figure 3.1.

For each state, varying numbers of collection sites were established. Spikelets harvested from each of the collection sites within a state were designated as an accession. An accession was identified as a group of harvested panicles within a natural population of *U. paniculata* in a state. More than one hundred accessions of *U. paniculata* were collected, kept in paper bags, and transported to the Department of Agronomy, Louisiana State University, Baton Rouge, Louisiana for threshing. Seeds were mechanically threshed and seeds were counted, sorted for quality and kept dry in small plastic vial containers (Table 3.3).

3.3 Seed germination and nursery grow-out

Germination tests were performed on March 2003 for all the accessions after seeds were threshed from the spikelets. Since seed supply of *U. paniculata* was very scarce, all available good quality seeds belonging to all accessions (Table 3.3) were used for germination. Plastic rectangular petri dishes (9.5 x 9.5 mm; Falcon®) were used for the germination experiment. Brown germination papers cut into rectangular shapes were placed onto the rectangular petri dishes. A total of 25 seeds per petri dish were placed and laid on the germination paper. A 0.05% fungicide solution of Vitavax 200® was used to moisten the germination paper to limit fungal growth. Vitavax 200® contains 17% carboxin and 17% Thiram at 2.06-2.74 g/kg (Gustafson Company; www.agsco-agdepot.com/infosheets). All petri dishes containing the seeds were placed in a germinator set at alternating temperature of 23^0 C and 34^0 C. Using a backlight board, germinated seeds were counted. In petridishes where fungal growth was visible (dark spots), anti-fungal solution was added. The dishes were kept moistened during the study. Regular monitoring of the germination set-up was done until seed germination was complete.

Table 3.3 *Uniola paniculata* L. accessions collected from southeastern Atlantic and Gulf coast areas and ID assignments.

State	Accession No.	No. of spikelets	Total seeds	Seed wt (g)	Seed/ gram	Bulk seed weight (g)	Estimated seed number
TX	01LA-01	273	406	0.55	229.7	1.816	823
TX	01LA-02	300	117	0.15	230.3	8.179	2001
TX	01LA-05	188	24	0.04	235.5	1.978	487
TX	01LA-09	288	259	0.28	283.5	17.418	5196
TX	01LA-12	300	61	0.06	299.7	3.314	1054
TX	01LA-13	300	82	0.07	357.1	3.195	1223
TX	01LA-17	211	229	0.24	281.7	6.753	2131
LA	01LA-15A	121	37	0.10	174.2	-	37
LA	01LA-15C	224	279	0.40	224.1	-	279
LA	01LA-15D	192	78	0.18	202.6	-	78
LA	01LA-15E	250	380	0.55	224.5	-	380
LA	01LA-15F	236	321	0.46	271.5	-	321
LA	01LA-15H	217	442	0.64	237.4	-	442
LA	01LA-15I	280	756	1.34	188.5	-	756
LA	01LA-15J	229	349	0.39	325.1	-	349
LA	01LA-15K	190	364	0.87	207.6	-	364
LA	01LA-15O	240	231	0.33	225.8	-	231
LA	01LA-15P	219	166	0.35	-	-	-
LA	01LA-15T	181	332	0.72	227.8		332
LA	01LA-15	291	222	0.31	242.8	82.172	20175
LA	01LA-16A	200	167	0.37	229.7	-	167
LA	01LA-16B	200	136	0.26	254.0	-	136
LA	01LA-16D	200	496	1.35	184.2	-	493
LA	01LA-16E	207	386	0.52	245.4	-	386
LA	01LA-16F	147	499	0.68	254.7	-	499
LA	01LA-16H	191	417	0.83	247.4	-	417
LA	01LA-16I	98	165	0.69	239.6	-	165
LA	01LA-16J	165	267	0.40	278.8	-	267

Table 3.3 continued

LA	01LA-16K	114	203	0.39	266.8	-	203
LA	01LA-16L	180	317	0.63	251.3	-	317
LA	01LA-16M	300	752	1.25	201.8	-	752
LA	01LA-16N	276	766	1.12	227.9		766
LA	01LA-16O	270	207	0.39	-	-	-
LA	01LA-16P	88	167	0.71	235.4	-	167
LA	01LA-16Q	158	140	0.22	301.5	-	140
LA	01LA-16R	100	110	0.48	228.5	-	110
LA	01LA-16S	169	297	0.60	228.8	-	297
LA	01LA-16T	217	177	0.25	266.3	-	177
LA	01LA-16	212	387	0.50	273.6	30.822	8820
LA	01LA-19	300	399	0.57	236.3	23.225	5886
AL	01LA-18	300	301	0.36	250.8	14.538	3947
AL	01LA-21	216	206	0.29	264.6	7.764	2260
FL	01LA-26	285	383	0.59	216.5	72.306	16039
FL	01LA-27	300	431	0.50	230.2	10.108	2758
FL	01LA-28	300	467	0.52	271.7	16.401	7923
FL	01LA-29	254	353	0.61	170.9	54.014	9584
FL	01LA-30	300	268	0.42	197.5	24.639	5133
FL	01LA-31	300	381	0.53	232.0	9.798	2654
FL	01LA-32	272	104	0.11	233.8	7.672	1898
FL	01LA-33	288	413	0.45	258.3	25.600	7024
FL	01LA-34	300	418	0.35	330.2	25.949	8987
FL	01LA-35	222	246	0.32	252.1	70.840	18105
FL	01LA-37	300	424	0.50	237.0	72.619	17627
FL	01LA-38	300	445	0.50	220.9	113.074	25421
FL	01LA-39	300	412	0.50	223.9	94.781	21638
MS	01LA-40	300	47	0.15	284.6	2.620	793
MS	01LA-41	250	69	0.28	252.7	3.582	974
MS	01LA-47	263	28	0.03	241.2	0.692	195

3.4 Nursery seedling grow-out

Seedlings from all the accessions germinated were immediately taken out from the germinator for nursery grow-out. Varying counts of seedlings from each accession were individually transplanted into Speeding® trays (10 x 6 holes) and labeled (60 seedlings per tray). The Speeding® trays were filled with Jeffy-Mix Plus® (Jiffy Products of America), soil, and sand (1:1:2).

All transplanted seedling in the trays were placed in the green house for grow-out (Figure 3.2). The greenhouse temperature was approximately 20^0 C under natural light conditions and photoperiod.



Figure 3.2 *Uniola paniculata* L. seedlings from approximately 100 accessions grown in Speeding® trays (10 x 6 holes) at the green house for phenotypic evaluation and molecular analyses.

3.5 Plant tissue source for Amplified Fragment Length Polymorphism (AFLP) analysis

All of the *U. paniculata* leaf tissues used for AFLP analysis were collected from the four-month old greenhouse grown *U. paniculata* seedlings (Table 3.3). Nineteen accessions were selected for AFLP analysis. Geographic distance and location of each accession within

states were also given a consideration. These accessions were selected to represent the eight states. The number of accessions per state was decided based on the number of surviving plants available in the greenhouse. Texas and Florida each have four representative accessions. Louisiana, Mississippi, Alabama, South Carolina and North Carolina each have two accessions while Virginia has only one accession. An accession uniformly consists of ten individual plants.

In all, one hundred and ninety plants were individually sampled for its leaf tissues. Leaf tissues collected from the selected accession were individually placed in re-sealable plastic bag and properly labeled. While at the green house, collected samples (individual and bulked) were immediately placed in a cooler filled with ice to prevent DNA degradation. In the laboratory, leaf tissue samples were stored in -80^0 C freezer (REVCO Co.) for future molecular analysis.

3.6 Amplified Fragment Length Polymorphism (AFLP) protocol

The AFLP technique is a random fingerprinting technique that may be applied to DNA of any origin or complexity. The technique differs importantly from other random fingerprinting techniques (Caetano-Anolles et al., 1991; Welsh and McClelland, 1990) by its robustness and reproducibility (Vos et al., 1995).

The AFLP technique uses generic AFLP primers that consist of one "common" part corresponding to the adapter and restriction site sequence, and a unique part corresponding to the selective bases. AFLP primers are named "+0" when having no selective bases, "+1" when having a single selective base, "+2" for having two selective bases, *etc.*

A limited set of AFLP primers is sufficient to create a large set of different primer combinations, which can provide a unique pattern of amplified fragments.

3.6.1 Laboratory equipment and materials

Necessary laboratory equipment and materials for AFLP analysis are listed in Appendix 3.1. Solutions necessary for AFLP are presented in Appendix 3.2 while for the denaturing polyacrylamide gel electrophoresis, solutions were prepared as shown in Appendix 3.3.

CHAPTER 4
ASSESSMENT OF GENETIC VARIATION AMONG SEA OATS (*UNIOLA PANICULATA*) ACCESSIONS AS REVEALED BY AFLP (AMPLIFIED FRAGMENT LENGTH POLYMORPHISM)

4.1 Introduction

The ability to analyze genetic diversity among the plant populations has significant applications in conservation and breeding programs. Understanding genetic diversity is a prerequisite in quantifying germplasm resources. With this rationale, assessment of the amount and richness of genetic resources in any geographic locations is fundamental to the long-term goal in plant breeding, conservation of germplasm and its sustainable management.

In plant species, the level and structure of genetic diversity is influenced by evolutionary factors such as mutation, recombination, migration, genetic drift and selection (Sales et al., 2001). Estimates of genetic variability in population genetics, for example, are frequently used to measure the adaptability of one population to selective pressures common in a rapidly changing environment (Hey, 1997). Assessment of genetic diversity is significant not only for finding unique combination of genes but also in estimating loss of gene complexes, which suggests genetic erosion (Schaal et al., 1998). Therefore, the measurement and monitoring of genetic diversity becomes indispensable to the continuous viability of individuals and population genetic resources. The existing genetic diversity of land races has enabled humans to exploit plant genetic resources to increase food supply by generating better cultivars through conventional breeding in the early days (Hayes et al., 2000). New alleles have been discovered through naturally occurring mutations and more adaptable plant materials have been produced. Breeders were able to generate genetic variation to improve cultivars using Mendel's hybridization principles. On the other hand, rapid fixation of traits in

gene pools due to mutation have also made natural selection for subtle difference more difficult in both wild and cultivated populations (Stuber et al., 1999). For this reason, molecular marker systems have found to be a useful tool in screening unique traits. Likewise, their utilization has been found equally indispensable in basic diversity assessment of natural resources.

Genetic diversity research is shifting away from traditional inventory surveys as molecular marker technology became available. Advances in molecular biology have resulted in the development of a number of powerful new diagnostic tools for investigating genetic variation in plants and animals. These tools, (*e.g.* RFLP, RAPD, AFLP *etc.*) have substantially complemented the previously used techniques such as isozymes (Caetano-Annolles, 1997).

Uniola paniculata belongs to the grass family Poaceae and is commonly known as sea oats. Sea oats thrive in the unstable, xeric environment of the dune community exposed to regular salt spray. It produces a well-developed horizontal root system that extends throughout the new sand deposits with stems sprouting from the node along the rhizomes. Newly established roots normally produce shoots and form clumps of leaves with panicles (Figure 4.1). It is among the most effective native sand-binding grasses and an important contributor to dune formation and stabilization. It has the ability to grow best under the low soil-moisture conditions. Their extensive rhizome system (Figure 4.1) helps stabilize the sand formation around them while their stem growth acts as a windbreak.

New sea oat populations are established by sexual reproduction. Flowers are produced and fertilized by wind-dispersed pollen. Fully developed fertilized seeds are dispersed by the wind and distributed along the sand dunes. The seeds remain dormant

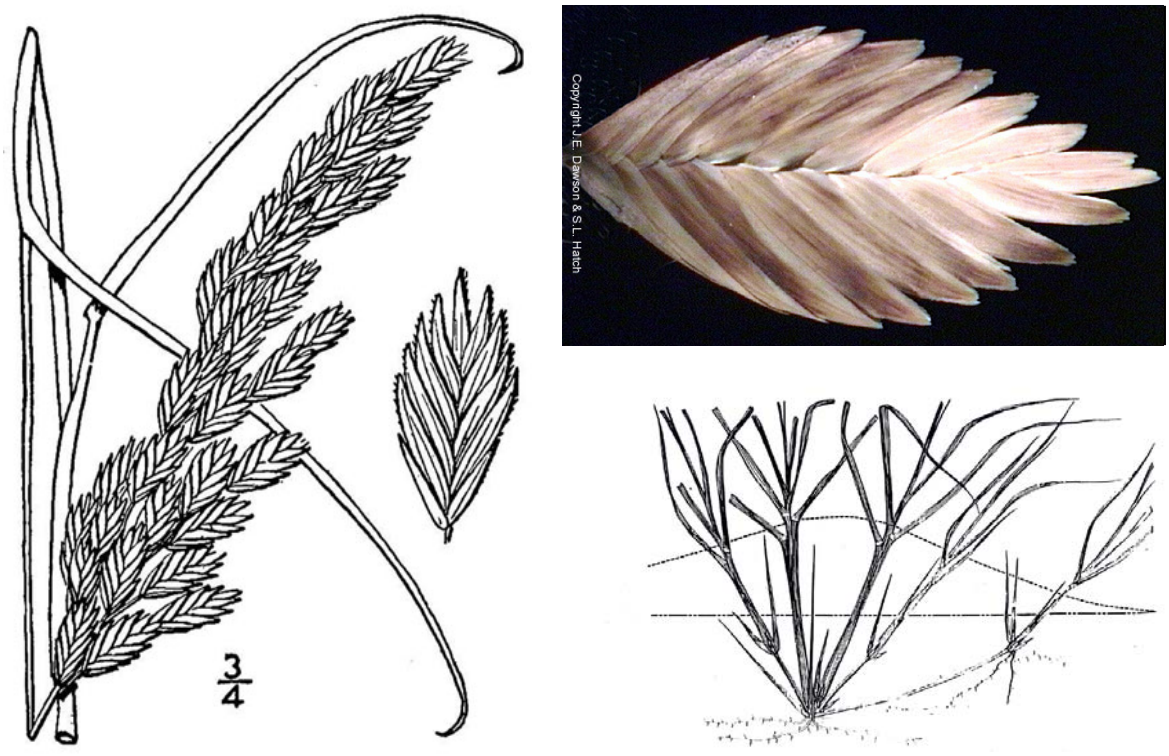


Figure 4.1 Schematic diagram of *Uniola paniculata* L. showing the spikelet (above right) and rhizome system (below right).

during winter and germinate in spring (Westra and Loomis, 1966). The highest percentage of germination is observed to occur in areas where seeds are buried under 2.5 to 5.1 cm of sand (Woodhouse and Hanes, 1966). During the first two months, the root system develops rapidly and normally extends 10 times longer than its shoot height. The extensive root system enables the sea oats seedlings to extend farther and thus, stabilization of the sand dune takes place.

Given the ecological importance of *U. paniculata* and its role in coastal dune ecosystem, it is important to evaluate its genetic diversity. Accessions of *U. paniculata* gathered from its natural population in southeastern Atlantic and Gulf coasts were characterized at DNA level using the AFLP (amplified fragment length polymorphism) technique.

The choice of AFLP is based on its particular strength to generate high volume markers in a short time without the need of prior sequences and in its capability to resolve highly distinct polymorphisms in a wide variety of plant species (Vos and Kuiper, 1997). AFLP selectively amplify a subset of genomic restriction fragments by polymerase chain reaction (PCR). DNA is digested with restriction endonucleases. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotides extending into the restriction fragments are added to the 3' ends of the PCR primers such that only a subset of the restriction fragments are recognized. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The subsets of amplified fragments are analyzed by denaturing polyacrylamide gel electrophoresis to generate the fingerprints. The polymorphisms produced by AFLP are typically inherited in Mendelian fashion and may, therefore, be used for genotyping and genome mapping.

The versatility of AFLP has been demonstrated in several studies on molecular genetic diversity. Individual assay based on six AFLP primer combinations distinguished nineteen accessions of cashew (*Anacardium occidentale*) (Archak et al., 2003). The level of AFLP polymorphism resolved noticeable amounts of intraspecific genetic variation among Texas bluegrass (*Poa arachnifera*), and identified parental lines from true hybrids (Renganayaki et al., 2001). In assessing taxa inter-relationship, hybrid plants origin and overall variation in its collection core, Hodkinson et al. (2002) utilized AFLP for characterizing European grass (*Miscanthus sinensis*). Results from 998 polymorphic AFLPs have detected intra-specific variation and distinctly separated *M. sinensis* collection into subdivision. Evolutionary relationship of 146 Azuki accessions from 6 Asian countries was

assessed using AFLP and a clear clustering of distinct accessions sharing 7 evolutionary groups was obtained (XuXiao et al., 2003). In perennial forage Rhodesgrass (*Chloris gayana*), 237 AFLP markers revealed the major proportion of the total genetic variation occurred within cultivars from East and South Africa and cluster analysis grouped cultivars based on its geographic origin (Ubi et al., 2003). AFLP assays to evaluate genetic diversity in 58 accessions of jointed goatgrass (*A. cylindrica*) and 6 accessions of the related wild species barb goatgrass (*A. triuncialis*) were performed by Pester et al. (2003). On a subset of the 58 jointed goatgrass accessions from the US and Eurasia, AFLPs distinguished among all but 2 of the 16 accessions (Pester et al., 2003).

The genetic diversity produced by AFLP for 94 genotypes of wild barley (*Hordeum spontaneum*) from ten different locations in Israel was studied by Turpeinen et al. (2003). 189 polymorphic markers (8 primer combinations) produced 204 discernible loci that discriminated each genotypes unique banding profile and resulted in a genetic similarity coefficient between 0.74 and 0.98. Despres et al. (2003) described AFLP as a powerful tool that resolved the complex genetic relationships between the morphological entities constituting the genus *Trollius*. The author noted that although 76% of the total AFLP variability was found within a priori defined morphological groups, the variability differentiating groups was adequate to create congruent cladistic and phenetic trees. A high degree of diversity was established among the Spanish olive variety by using AFLP (106 polymorphic bands; six primer combinations) where the analysis of 38 accessions resulted in 10 distinct clusters that correspond to named variety designations. Similarity among varieties ranged from 0.60 to 0.72 (Sanz-Cortes et al., 2003).

AFLP being a technique for DNA fingerprinting, its usefulness in this present study is on determining the genetic structure of *U. paniculata* plants based on its similarity and if significant genetic variation exists within and among the accessions. AFLP fingerprinting of these accessions would help measure genetic diversity of *U. paniculata* collected from southeastern Atlantic and Gulf coasts.

4.2 Materials and methods

4.2.1 Plant materials

Nineteen selected accessions of *U. paniculata* representing eight different states of the southeastern Atlantic and Gulf coasts were utilized for amplified fragment length polymorphism (AFLP) analysis. Ten individual plants were sampled for each accession. The number of accessions for each state varied due to low survival of seedlings belonging to certain states. Table 4.1 shows the accessions and origin. All plant materials used for this study were approximately four-month old plants grown in the green house.

Leaf tissues from 190 plants were collected for DNA extraction. Leaf tissue from each plant was harvested separately, packed in sealed plastic bags and labeled according to their accession assignment. All collected leaf tissues were immediately placed on ice to avoid DNA degradation and the stored in -80^0 C freezer (REVCO Co.) in the laboratory until DNA extraction.

4.2.2 Genomic DNA extraction

Extraction of genomic DNA from young leaf tissue samples was done for each of the 190 individuals. Leaf tissue samples were ground into a fine powder in liquid nitrogen using a mortar and pestle. Up to 100 mg of the powder were transferred to a microcentrifuge tube and kept in the freezer at -80^0 C until use.

Table 4.1 Selected *U. paniculata* L. accessions for AFLP analysis and collection sites within each state.

Accession number	Accession name	Collection site (Source)	State
1	01LA-02	Hwy 87 Bolivar Peninsula, Flake	Texas
2	01LA-05	Hwy 87 Follets Island, SW Toll Bridge	Texas
3	01LA-09	Hwy 53 NE Newport Pass Gulf # 6	Texas
4	01LA-17	North Padre Island	Texas
5	01LA-15	Fourchon Beach Plots	Louisiana
6	01LA-16	Fourchon Beach Plots	Louisiana
7	01LA-41	West Ship Island	Mississippi
8	01LA-47	Petit Bois Island	Mississippi
9	01LA-19	Dauphin Island, West	Alabama
10	01LA-21	Gulf Shores	Alabama
11	01LA-29	Eglin AFB, Sta. Rosa Island	Florida
12	01LA-33	Henderson Beach	Florida
13	01LA-35	Tyndall AFB, West Crooked Island	Florida
14	01LA-39	Perdido Key	Florida
15	01NC-15	Debedieu Beach	South Carolina
16	01NC-19	Prince George	South Carolina
17	01NC-1	Atlantic Beach	North Carolina
18	01NC-11	Sunset Beach	North Carolina
19	01LA-53	Assateague Island	Virginia

Good quality genomic DNA was extracted using the GenElute™ Plant Genomic DNA kit (Sigma-Aldrich®). The kit contains all the reagents, columns, and tubes necessary to isolate genomic DNA. Following the instruction provided by the manufacturer, leaf tissue cells were lysed with 350 µl of lysis solution A and 50 µl of lysis solution B in the microcentrifuge tubes. After vortexing, the samples were incubated at 65^0 C for 10 minutes with occasional inversion to dissolve the precipitate. No RNA treatment was done since the kit is designed to selectively isolate DNA.

A precipitation solution (130 µl) was added to the mixture, completely mixed by inversion and incubated on ice for 5 minutes. The samples were centrifuged (12,000-16,000 x g) for 5 minutes. Debris was filtered by pipetting the supernatant onto the filtration column and centrifuged at 30,000 x g for 1 minute. The flow-through liquid was collected and added with 700 µl of binding solution. Binding of DNA into the column membrane was achieved by adding 500 µl of the column preparation solution and centrifuging (12,000 x g for 1 minute). DNA bound to the column membrane was washed twice with ethanol mixed in the wash solution concentrate and centrifuged at maximum speed for 3 minutes to dry the column. Pure genomic DNA was eluted into the collection tube by adding 100 µl of pre-warmed (65^0 C) elution solution to the column and centrifuged at maximum speed for 1 minute. Extracted genomic DNA samples were stored in freezer at 4^0 C (VWR Scientific).

The purity and quality of genomic DNA was determined by running a 1% agarose (Gibco® BRL, Carlsbad, CA.) gel. Genomic DNA quantity was determined by staining small aliquot of extracted DNA from each plant with diluted Ethidium bromide solution. Intensities of the samples were compared to known concentrations of λ DNA (50, 100, 200 ng) under the ultraviolet (UV) light. The extracted DNA samples were standardized at recommended concentration of 350-450 ng per µl for AFLP analysis.

4.2.3 Amplified Fragment Length Polymorphism (AFLP) analysis

The amplified fragment length polymorphism (AFLP) protocol was followed as described by Vos et al. (1995). AFLP reactions involved the following steps: (1) digestion, (2) ligation, (3) pre-selective amplification, and (4) selective amplification. Optimized concentration of genomic DNA (350-450 ng per µl) from all of the one hundred ninety individual plants were used.

4.2.3.1 Genomic DNA digestion

It is important that DNA preparations need to be of high quality to allow complete digestion by the restriction enzymes. It should be noted that much often contaminating agents are co-purified together with the DNA and when very little DNA is present; the ratio of contaminants over DNA may be such that it interferes with digestion. Only 50 ng of DNA is needed for AFLP template preparation. DNA concentrations can be determined by measuring OD_{260}.

For this study, running of a small aliquot of DNA on a 1% agarose gel was done. The agarose image allowed inspecting the integrity of the DNA quality and concentration.

Digestion/restriction was achieved in a PTC-100 Programmable Thermal Controller (MJResearch, Inc., Waltham, Mass.) programmed for 2 hours at 37^0 C followed by 15 minutes at 70^0 C and hold at 4^0 C. Digestion/restriction was done with restriction cocktail prepared with the following components in a 30-µL reaction (Table 4.2).

Table 4.2 Restriction mix preparation for 1 reaction.

Ingredients	1x reaction
*Eco*RI (12 units per µL) (Invitrogen)	0.4
*Mse*I (10 units per µL) (New England BioLab)	0.5
5x Reaction buffer (µL) (LabMade)	6.0
Sterile water (µL)	8.1
Genomic DNA (350-450 ng per µL)	15.0
Total volume (µL)	30.0

4.2.3.2 Adapter preparation

Two primers (17-mer and 15-mer) were diluted into 100 pmol/µl with sterile water to prepare the *Mse*I adapter (5 pmol per µl). Equal volume of 100 µl 17-mer primer and 100 µl 15-mer primer were then diluted with sterile water in a final volume of 1000 µl.

4.2.3.3 Adapter ligation of digested/restricted DNA

Ligation of adapter to the digested DNA was followed. Ligation mix was prepared first in a 30-µL reaction and added with the digested/restricted DNA. The components comprised the ligation mix as provided in Table 4.3. Adapter ligation was performed for 3 hours at 20^0 C in a PTC-100 Programmable Thermal Controller (MJResearch, Inc., Waltham, Mass.). Ligated DNA products were diluted (10x) for preselective-amplification step.

Table 4.3 Ligation mix preparation for 1 reaction.

Ingredients	1x reaction
*Eco*RI-adapter (50 pMol per µL)	1.0
*Mse*I-adapter (5 pMol per µL)	1.0
T4 DNA ligase (5 units per µL) (Invitrogen)	0.2
5x Ligation buffer with ATP (µL)	6.0
Sterile water (µL)	1.8
Digested DNA (µL)	20.0
Total volume (µL)	30.0

Restricted DNA was incubated for a total of 3 hours with restriction enzymes and in the presence of T4 DNA ligase and oligonucleotide adapters. The adapters do not restore the restriction sites so, the presence of the restriction enzymes in the ligation step results in almost complete adapter-to-fragment ligation because of the restriction of fragment formed in ligation. On the other hand, prolonged incubation with restriction enzymes is not recommended because of possible "star" activity of *Eco*RI giving reduced cleavage specificity and ultimately aberrant AFLP fingerprints.

4.2.3.4 Pre-selective amplification

Primers used in this step consisted of a core sequence, a restriction specific sequence and a selective single-base extension at the 3'end. The sequences of the adapters and restriction sites serve as primer binding sites for the pre-selective amplification. Each pre-

selective primer has a nucleotide that recognizes the subset of restriction fragments having the matching nucleotide downstream from the restriction site.

The primary products of the pre-selective PCR are those fragments having *Eco*RI and *Mse*I ends. Only those fragments with primer sites complementary to the additional bases will amplify. Diluted ligated DNA products were preamplified in a 30-µL pre-selective amplification reaction mix consisting of the given components (Table 4.4).

Table 4.4 Pre-selective amplification mix preparation for 1 reaction.

Ingredients	1x reaction
*Eco*RI-Pre primer (µL)	1.0
*Mse*I-Pre pimer (µL)	1.0
10x PCR buffer (µL)	3.0
25 mM $MgCl_2$ (µL)	0.6
2 mM dNTP (µL)	3.25
Taq polymerase (µL)	0.2
Sterile water (µL)	15.95
Ligated DNA (µL)	5.0
Total volume (µL)	30.0

Pre-selective amplification in PTC-100 Programmable Thermal Controller (MJResearch, Inc., Waltham, Mass.) was set at 94^0 C for 30 seconds, 56^0 C for 1 minute and 72^0 C for 1 minute. Thirty cycles were performed followed by hold at 4^0 C. After pre-selective amplification, the pre-amplified DNA was diluted (5x) with sterile water sufficient for several selective amplification using different primer combinations of *Eco*RI and *Mse*I.

4.2.3.5 Selective amplification of templates

Working with AFLP reaction mixes is important for the reliability and reproducibility of AFLP reactions. All throughout the reaction preparation, mixes were placed on ice to lower the activity of the *Taq* polymerase.

The template DNA was pipetted last and mixed well with the selective amplification reagents. After mixing of the reagents, the AFLP reaction was started as soon as possible.

Selective primers consisted of an identical sequence to the pre-selection primers plus three additional selective nucleotides at the 3'end. Different primer combinations would generate different sets of banding patterns. Selective amplification was done in a 12-µL reaction prepared with the components listed in Table 4.5.

Table 4.5 Selective amplification mix preparation for 1 reaction.

Ingredients	1x reaction
*Eco*RI-primer+3 (µL)	0.5
*Mse*I-pimer+3 (µL)	0.5
10x PCR buffer (µL)	1.25
25 mM $MgCl_2$ (µL)	1.2
2 mM dNTP (µL)	1.5
Taq polymerase (µL)	0.05
Sterile water (µL)	4.35
Preamplified DNA (µL)	3.0
Total volume (µL)	12.0

Selective amplification in PTC-100 Peltier thermal cycler (MJResearch, Waltham, Mass.) was programmed as follows: cycles 1 for 30 seconds at 94^0 C, 1 minute at 65^0 C, 1 minute at 72^0 C; cycles 2-13: annealing temperature was reduced by 0.7^0 C in every successive cycle; cycles 14-36: annealing at 56^0 C; final extension for 5 minutes at 72^0 C and hold at 4^0 C. Amplified DNA products were stored in 4^0 C freezer.

Diluted (5x) preamplified DNA products was selectively amplified using the twelve *Eco*RI+*Mse*I primer combinations.

The 12 primers used in the selective amplification of the preamplified templates were then selected.

4.2.4 Denaturing polyacrylamide gel electrophoresis of AFLP products

The AFLP reaction products were analyzed on 6% denaturing polyacrylamide gels. These gels were essentially normal sequencing gels (Maxam and Gilbert, 1980) with the exception that a lower percentage of polyacrylamide was used. Ingredients for gel preparation were Urea (50.4 g), 40% Acrylamide:Bis (19:1) solution (18.0 ml), 10x TBE buffer (6.0 ml), and distilled water (54.0 ml). Six hundred microliters of 20% Ammonium persulfate (APS) and 100 µL of TEMED were added to the solution right before casting.

A BioRad Sequi-Gen® (38 x 50 cm) unit was used to run the 6% denaturing polyacrylamide gel. Preparation of the sequencing gel-casting unit (outer glass plate and the integral plate chamber) was done meticulously. The outer glass plate was properly cleaned of any debris, air-dried and treated with 2 µL of bind silane (0.1 µL Sigma® bind silane in 10 ml of 0.5% acetic acid and 95% ethanol) on one side. The integral plate chamber was treated with 2 ml of repellant solution (SigmaCote®). The silane treatments cause the gel to stick to the outer glass plate upon disassembly of the gel cassette after electrophoresis. The Sequi-Gen® sequencing gels require about 120 mL of gel solution. The gels were casted at least 2 hours before use and pre-run for 1 hour before loading the samples (0.5x TBE was used as running buffer). The purpose of pre-running was to "warm up" the gel to about 50^0 C. This temperature was maintained throughout electrophoresis. This warrants an even heat distribution during electrophoresis and is crucial for good quality fingerprints. The denaturing polyacrylamide gel electrophoresis was run for 100 minutes at 50^0 C, 110 W (BioRad PowerPac 3000).

AFLP reaction products (12 μL) were added with an equal volume of loading dye. Denaturing of the samples was done for 4 minutes at 95^0 C immediately before loading and placed on ice while loading into the gel.

4.2.5 Silver staining of the polyacrylamide gels

Silver staining of the gels follows a series of steps done manually. The following solutions (Appendix 4.1) were prepared separately and placed in rectangular plastic: (a) fix/stop solution containing 200 ml Acetic acid mixed with 1800 ml of distilled water; (b) silver stain solution containing 3 ml Formaldehyde and 2 g Silver nitrate dissolved in 2000 ml nanopure water; and (c) developer solution containing 60 g of Sodium carbonate dissolved in 2000 ml nanopure water. The developer solution was chilled at 20^0 C and added with 3 ml Formaldehyde and 40 mg Sodium thiosulphate (200 μL of 20 mg per ml stock).

After electrophoresis, the outer glass plate with the gel on it was placed in a container filled with fix/stop solution for 15-30 minutes with continuous shaking until dyes were no longer visible. The gel was washed with distilled water for 10 minutes and then transferred to the container filled with silver staining solution and continuously shaken for 20 to 30 minutes. The gel was rinsed with nanopure water for 10 seconds was and then immediately placed into the pre-chilled developer solution (added with Formaldehyde and Sodium thiosulphate) until AFLP bands appeared. The developed gel was then placed into the fix/stop solution for 3 minutes followed by washing with distilled water for another 3 minutes. A sheet of 3 mm Whatmann white paper was placed on top of the developed gel to visualize the AFLP bands. As an alternative, the gel was soaked in 2% NaOH solution, lifted from the plate with the help of a sheet of Whatman paper, scanned directly using a regular scanner, and saved the image in JPEG format.

4.2.6 AFLP scoring and data analyses

Silver stained of the twelve primer combinations for all of the one hundred ninety plants were scored for presence ("1") or absence ("0") of bands. Binary matrices were made for statistical analyses using NTSYSpc (Numerical Taxonomy System) version 2.10t (Rohlf, 1997). Dice (1945) coefficient of similarity was used to estimate similarity among plants within accessions, accessions within states, and among states. The similarity coefficients derived from Dice (1945) is similar to the Jaccard's similarity coefficient but Dice similarity coefficients give twice the weight of the matching present bands, where; "1" = presence of band and "0" = absence of band). Using this formula, 2a/ (2a+b+c) where; a = the number of matching present bands ("1" and "1"), b = the number of unmatched bands ("1" and "0"), and c = the number of unmatched band ("0" and "1"), the Dice similarity coefficients were computed.

Similarity coefficients were used to cluster accessions with the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973), a hierarchical method of clustering. Sequential agglomerative hierarchical nested cluster analysis (SAHN) is a bottom-up clustering method where clusters have sub-clusters. This analysis starts with every single object and in every successive iteration and agglomerates (merges) the closest pair of clusters by satisfying the Dice similarity criteria until all the data is grouped in a cluster. The tree or dendrogram produced from the agglomerative hierarchical clustering analysis is a close reflection of the agglomerative algorithm that generated the clusters.

Dice similarity coefficient was used to create the genetic distance matrix (1-Dice similarity coefficient) following the SIMQUAL (similarity of qualitative data) procedure. SIMQUAL computes for the similarity of tested individual based on the binary data. A

matrix of cophenetic values was calculated by the ultrametric distance method from the genetic distance matrix to check how good the clustering result represents the original genetic distance. Ultrametric distance corresponds to the type of relationship of cluster groups projected in the dendrogram where similarity distance between tested individuals is computed.

Using the MXCOMP (matrix comparison) module, a cophenetic correlation value was computed (Sneath and Sokal, 1973) to test the goodness of fit or the correlation of the two matrices (cophenetic and tree matrix). Further representation of relationships among accessions was carried out using principal component analysis (PCA) on the original AFLP matrix data using the following modules of the NTSYS program: SIMQUAL (similarity of qualitative data), EIGEN (eigenvectors), and PROJ (projection). EIGEN is used to compute eigenvalues and eigenvectors for symmetric matrix such the correlation matrix while PROJ is utilized to project the measured variables onto one or more factor axes. PCA performs a common principal component analysis to fit a common set of principal component axes to two or more covariance matrices. Although principal component analysis is not necessarily suited for use with binary data, it is often used as a supplemental means of visualizing the relationships of the genetic profiles (Demeke and Adams, 1994).

Analysis of molecular variance (AMOVA) was applied using ARLEQUIN version 2.0 (Schneider et al., 2000) among individual plants, among accessions and among states to determine the distribution of genetic variation sources. Originally developed for population genetics (Excoffier et al., 1992), ARLEQUIN computes for molecular variance and presents a pairwise F-statistic for which the probability is tested by permutation analysis. Genetic variation was partitioned within and among plants, accessions and states. Significant values

assigned to variance components were based on 1000 random permutations of 190 individual samples assuming no genetic structure.

4.3 Results and discussion

4.3.1 AFLP marker diversity and polymorphism

AFLPs were used to characterize the genetic diversity of *U. paniculata* collected from nineteen different geographic locations in eight different states (Table 4.1). The efficiency of primer combinations was tested so that sufficient amount of polymorphism essential to infer genetic similarity of *U. paniculata* could be achieved. This was carried out by screening more than 24 combinations. Four plant DNA samples were used to test the 24 primer combinations and determined the primer combinations with most polymorphic bands. Twelve primer combinations were selected after the preliminary screening of *Eco*RI+3 and *Mse*I+3 primers.

The number of AFLP bands generated for each selected primer combination is presented in Table 4.6. The number of polymorphic markers was sufficient to discriminate variation among the test samples (Figure 4.2). Several studies have noted that the range of polymorphic bands generated in this study is adequate when using AFLP to characterize plant genetic resources (Archak et al., 2003; Segovia-Lerma et al., 2003; Hodkinson et al., 2002; Manubens et al., 1999). In this study, a total of 703 AFLP bands were generated across all plant samples. Among these, 417 bands were polymorphic with a polymorphism rate ranging from 42-81% and a mean of 59%. The highest polymorphic rate at 81% was observed in primer combination *Eco*RI-CAG+*Mse*I-CGA followed by *Eco*RI-ACT+*Mse*I-CTC and *Eco*RI-CAG+*Mse*I-ACG at 76% and 73%, respectively. Three primer combinations (*Eco*RI-AGG+*Mse*I-CAC, *Eco*RI-CAG+*Mse*I-CCT and *Eco*RI-CAA+*Mse*I-CCT) produced

Table 4.6 The number of bands and degree of polymorphism revealed by each AFLP primer combination.

Primer combination	Total bands	Polymorphic Bands	Polymorphism rate (%)
*Eco*RI-AGG+*Mse*I-CAC	68	29	42
*Eco*RI-AGG+*Mse*I-CCT	60	30	50
*Eco*RI-AGG+*Mse*I-CGT	64	36	67
*Eco*RI-AGG+*Mse*I-CGA	58	33	57
*Eco*RI-ACT+*Mse*I-CTC	97	73	75
*Eco*RI-ACT+*Mse*I-GAC	48	28	58
*Eco*RI-ACT+*Mse*I-CTG	58	33	57
*Eco*RI-CAG+*Mse*I-ACG	55	40	73
*Eco*RI-CAG+*Mse*I-CCT	40	17	42
*Eco*RI-CAG+*Mse*I-CGA	69	56	81
*Eco*RI-CAA+*Mse*I-CCT	52	22	42
*Eco*RI-CAA+*Mse*I-CGT	34	20	59
Total	**703**	**417**	**mean = 59**

only 42-43% polymorphic bands. Overall, the level of polymorphism as resolved by the twelve primer combinations is adequate to obtain a good genetic assessment. Segovia-Lerma et al. (2003) pointed out that at least 200 markers were required to adequately sample the genome of alfalfa and to achieve 95% chance of locating one known marker on the chromosome. It was indicated that results from the use of fewer primer combinations were consistent with the expected phylogenetic relationship (Segovia-Lerma et al., 2003). Our study agrees with the observation of Segovia-Lerma et al. (2003), as results of the cluster analyses from the use of single primer combination did not provide a significant genetic structure of the plants with regards to their geographic origin. Although there is no established minimum number of primer combinations to use, a reasonable number of primer

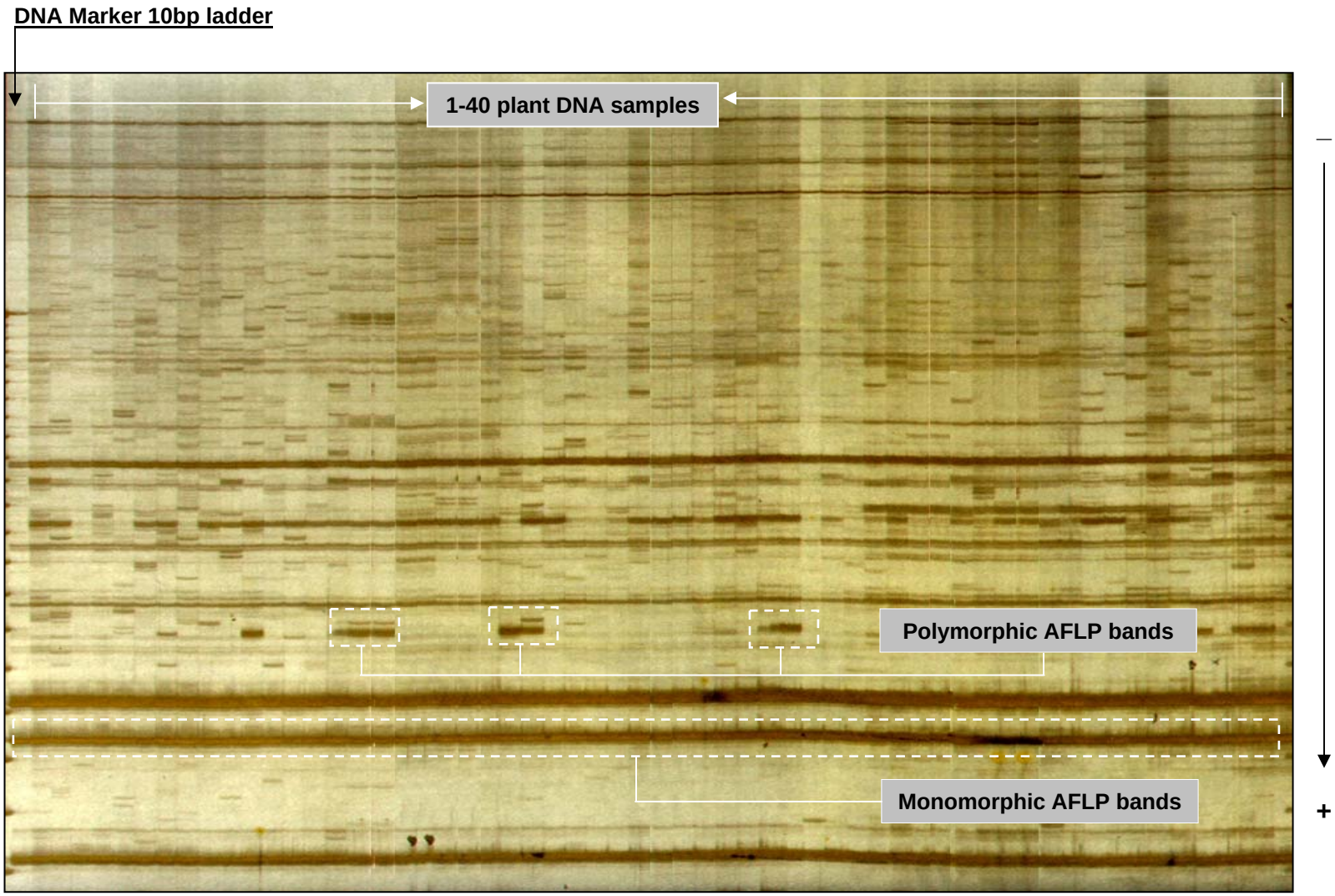


Figure 4.2 AFLP fingerprints of individual plant as resolved the 6% denaturing polyacrylamide gel (PAGE) after silver staining. PAGE was run at 50^{o} C for 100 minutes, 110W.

combinations and good number of polymorphic bands conforming to other published studies have been utilized for this study.

4.3.2 Genetic structuring of *U. paniculata* based on AFLP fingerprints

The first step toward generating the patterns of genetic structure by AFLP fingerprints (polymorphic bands or profiles) was accomplished using some of the available programs like NTSYSpc (Numerical Taxonomy System) version 2.10t (Rohlf, 1997) and ARLEQUIN version 2.0 (Schneider et al., 2000). Software like those of PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2002), PHYLIP (the Phylogeny Inference Package) version 3.6 (Felsenstein, 2002) among others can also be used for ecological and diversity studies.

Cluster analysis discriminates AFLP fingerprints of the plants that possess some properties of coherence and isolation (Jardine and Sibson, 1971) where highly similar fingerprints are grouped together in such a way that the other groups are as dissimilar as possible. These similarity and dissimilarity can be established using the coefficient derived by Dice (1945) that searches matching bands from the pool of individual AFLP profile. When hundreds of fingerprints need to be differentiated, the complexity of assigning individuals into a certain cluster can be achieved by mathematical algorithm employed in NTSYSpc. Hierarchal algorithms produce dendrograms that classify fingerprints in a most appropriate representation of the data for confirmatory purposes.

In this study, 417 polymorphic bands were subjected for cluster analysis in NTSYSpc to agglomerate genetically similar *U. paniculata* plants based on its AFLP fingerprints. Using the unweighted pair-group method (UPGMA) and Dice coefficient of similarity, clusters and subclusters of genetically related plants were constructed into a tree-form, hereafter referred

to as dendrogram. Figure 4.3 shows the general overview of accession groupings and its genetic similarity. The dendrogram was developed based on the sequential agglomerative hierarchal nested cluster analysis (SAHN) that well defined the similar groups. Three major clusters (Group I, II, and III) with subclusters (I-A, I-B and I-C; II-A and II-B) have been formed which were distinctly consistent to its original source of collection site. Group I clustered those accessions from Texas, Louisiana, Florida and Virginia. Subcluster I-A has Texas (LA2, LA5, LA9 and LA17) and Louisiana (LA15 and LA16) accessions and joined by subcluster I-B from Virginia (LA53) accession. Meanwhile, two accessions from Florida (LA35 and LA39) were clustered into Group I as subcluster I-C. Within Group I, LA53, LA35 and LA39 plants were closely aligned with Texas and Louisiana accessions. Taking into account the geographic distance, it could have been unlikely without a detailed genetic fingerprint to weigh Virginia and Florida accession to be closely similar to the accessions from the two states. Accessions from Mississippi (LA41 and LA47), Alabama (LA19 and LA21) and Florida (LA29 and LA33) were clumped together in one distinct cluster (Group II). Group II had identified two subclusters (II-A and II-B). Subcluster II-A comprised the accessions from Mississippi (LA41 and LA47) and Alabama (LA19 and LA21) while subcluster II-B has the rest of the Florida's accessions (LA29 and LA 33). The order in which these accessions were grouped in the dendrogram indicates possible exchange of genetic materials among these neighboring states. Group III has two subclusters consisting of the accessions from South Carolina and North Carolina. Subcluster III-A had NC1 and NC11 accessions from North Carolina while subcluster III-B comprised the accessions from South Carolina (NC15 and NC19). In Figure 4.4, the genetic distances calculated from the similarity matrix generally fell into different clusters with short distances among plants

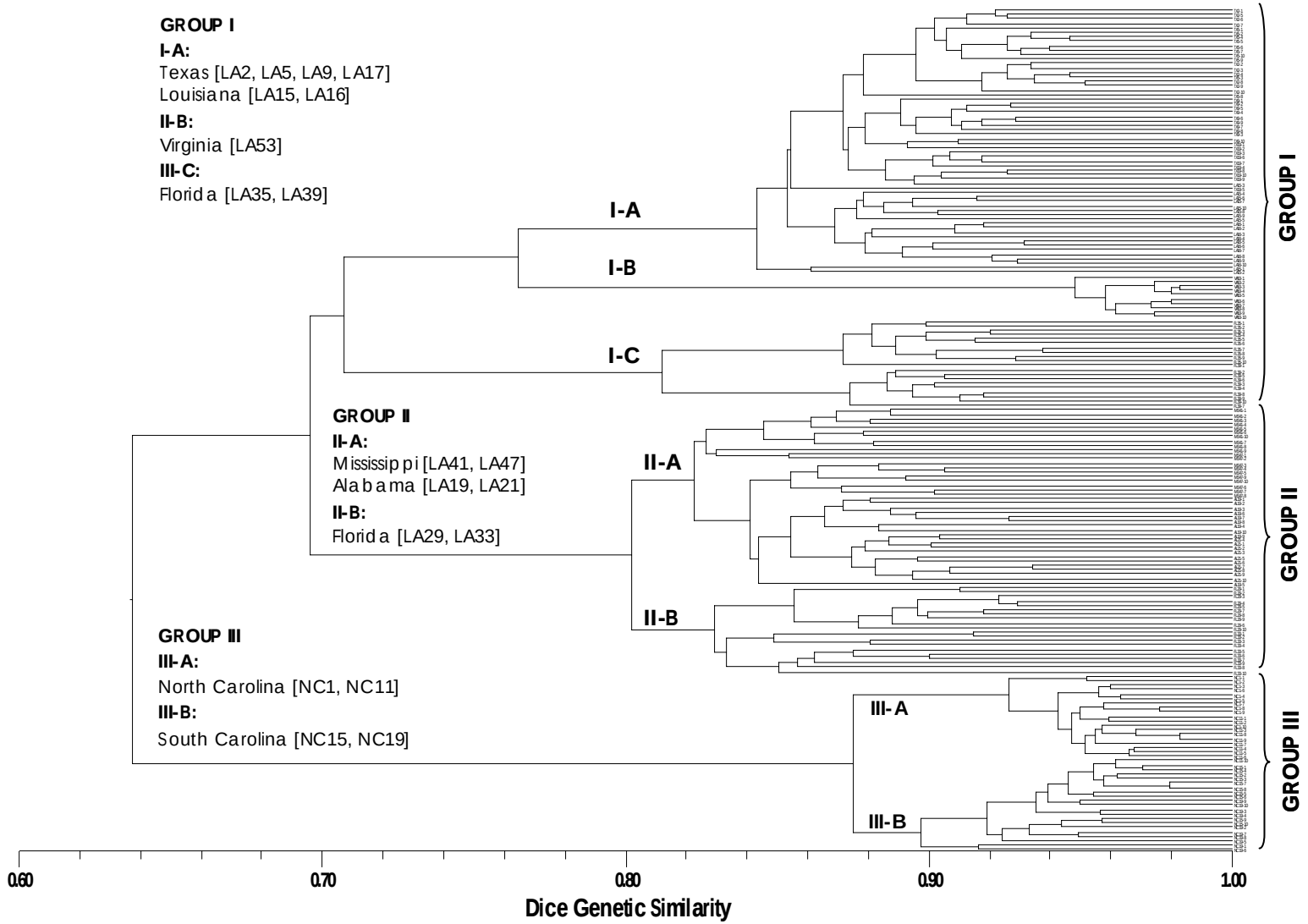


Figure 4.3 UPGMA dendrogram using Dice genetic similarity of 190 *Uniola paniculata* L. plants belonging to 19 accessions collected from 8 states: Texas, Louisiana, Mississippi, Alabama, Florida, South Carolina, North Carolina and Virginia. Group I has 3 subclusters (I-A, I-B and I-C) and Group II (II-A and II-B) and III (III-A and III-B) have 2 subclusters each.

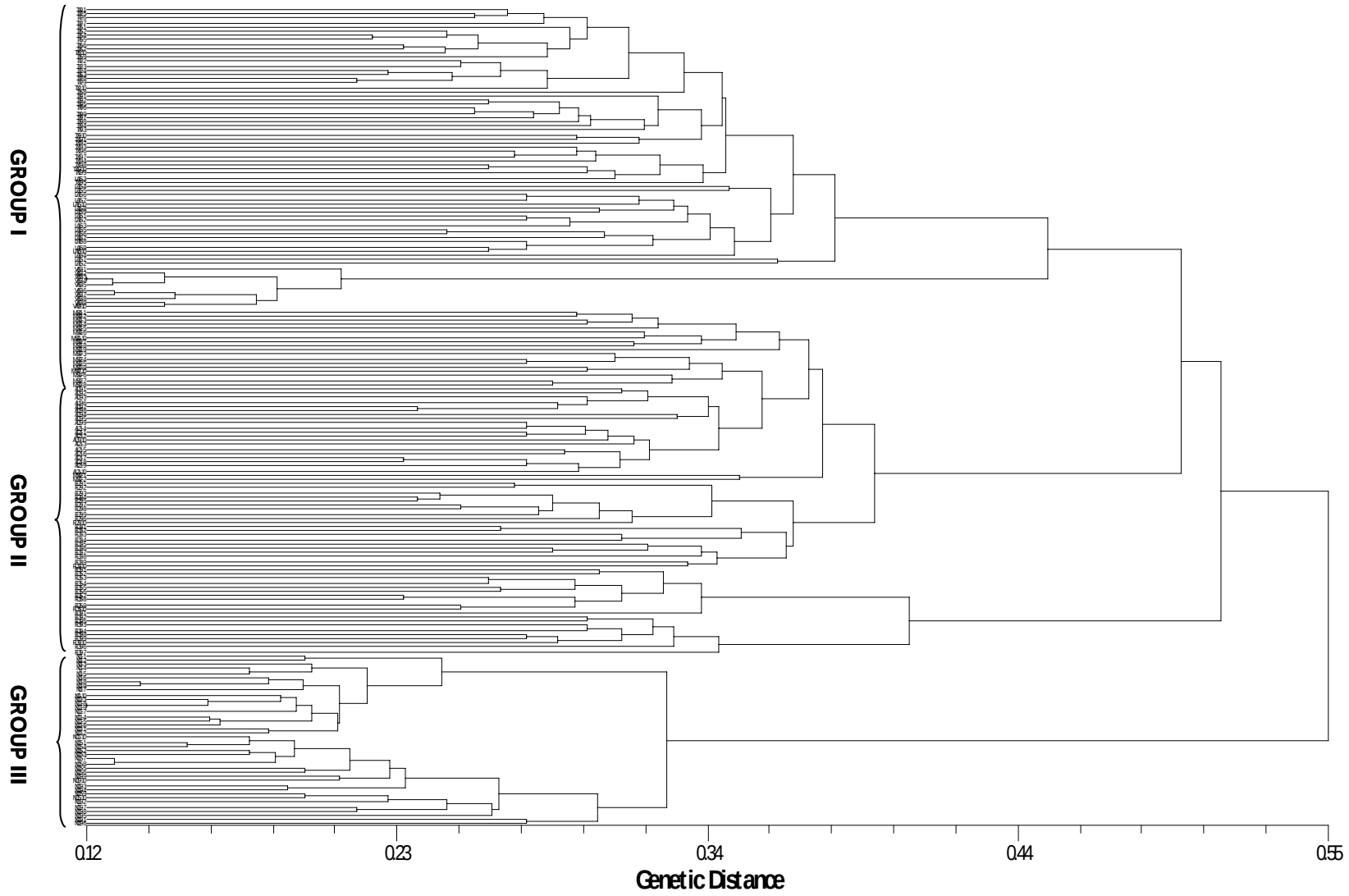


Figure 4.4 UPGMA dendrogram of genetic distance (1-Dice genetic similarity coefficient) of 190 *Uniola paniculata* L. plants belonging to 19 accessions collected from 8 states: Texas, Louisiana, Mississippi, Alabama, Florida, South Carolina, North Carolina and Virginia as clustered in Groups I, II and III.

within an accession and longer distances among accessions from different collection sites within the states. The collection sites within the state have some impact on the genetic distance among the accessions but the major genetic dissimilarity or variation reflected in the dendrogram occurred at the state level.

The extent of genetic similarity found among these nineteen accessions subsequently depicted the degree of polymorphism of the AFLP fingerprints. Genetic similarity across all plants was observed from 64% to 98% as calculated by the Dice similarity index. Average similarity among plants was computed from the genetic similarity coefficient matrix. Results show that average similarity of Texas and Louisiana plants were both at 88%. Virginia plants have a high average similarity of 96% while Florida plants were 80% similar. Mississippi and Alabama plants average similarity were 85% and 87%, respectively while South Carolina and North Carolina plants have an average similarity of 90%. Although the Virginia accession (LA53) was clustered (I-B) with Texas and Louisiana (I-A) in Group I, their similarity index was only 77%. Florida accessions (LA35 and LA39; subcluster I-C) clustered in Group I were 69% similar to I-A and I-B subclusters. In Group II, subcluster II-A comprising Mississippi (LA41 and LA47) and Alabama (LA19 and LA21) accessions were 83% similar while Florida (LA29 and LA33) was 84% similar. Subclusters in Group II (II-A and II-B) were 80% genetically similar. Group III subclusters (III-A and III-B) were 87% similar. At the major group level, Group II was 69% similar from Group I. Group III joined and was only 64% similar from Group II and I combined.

Cophenetic correlation result (goodness of fit) was used to determine the validity of the similarity matrix against the genetic distance for each plant within each accession (Figure 4.5). Result shows a high goodness of fit value at r=0.95 (p<0.001) indicating that 95% of

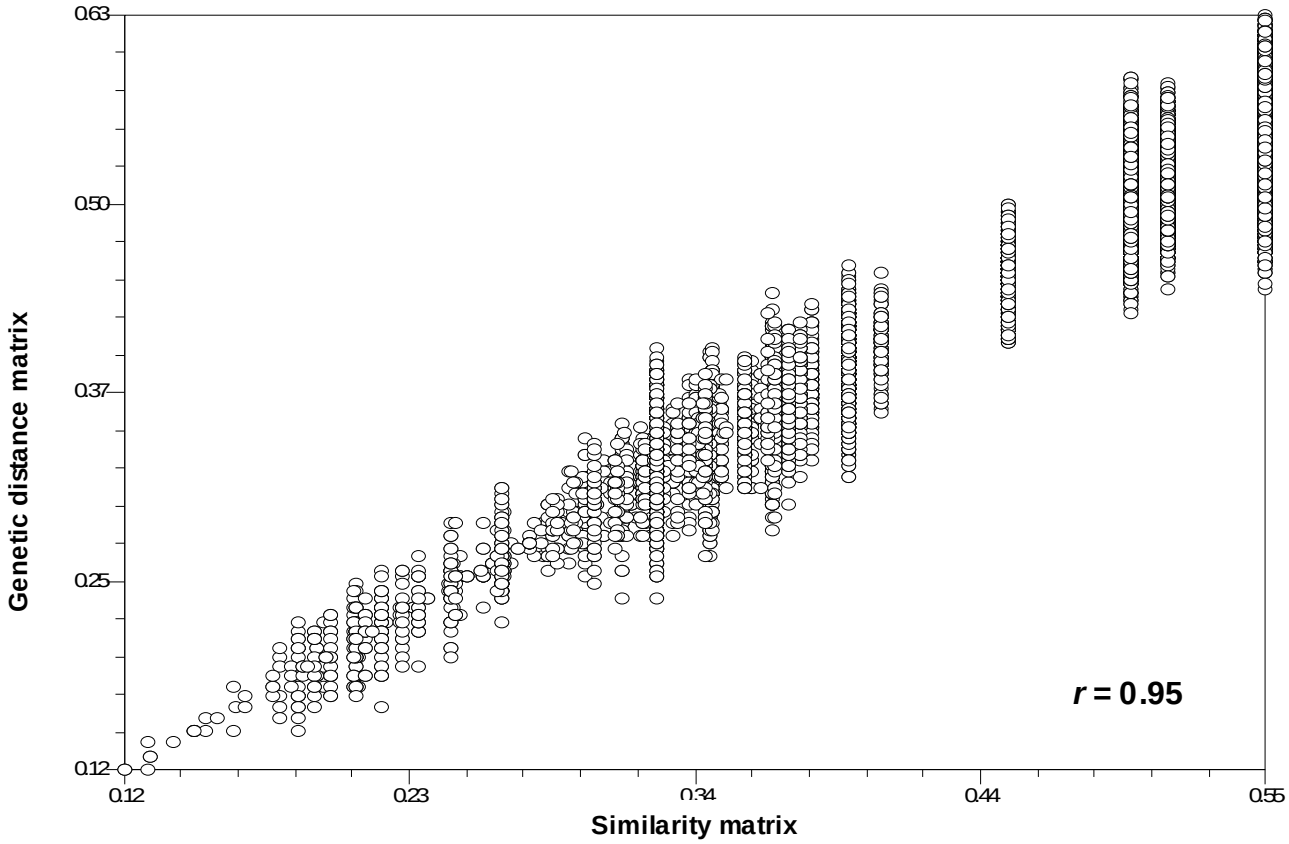


Figure 4.5 Matrix comparison analysis using ultrametric distance method to determine Mantel statistic Z test for association between the distance and similarity matrix. Cophenetic correlation (*r*) = 0.95.

the data were correctly explained by the cluster analysis. The normalized Mantel Z statistic for testing the equivalence of the similarity matrix based on the Dice coefficient estimated a mean correlation of 0.72 (p<0.001).

4.3.3 Principal component analysis

On the same data, principal component analysis (PCA) was done to visually validate the cluster analysis (Figure 4.6). The principal component analysis (Figure 4.6A) projects a similar number of groupings that confirm the result from the dendrogram. Following the distribution of the samples, the three major groups from the cluster analysis appeared to be well explained by the 2-dimensional components plotting PC1 and PC2 (Figure 4.6A). The plotted data were interpreted through the closeness of the angles from the origin and found out that the accessions were separated generally by small angles implying a close relationship among the grouped accessions. At the top right quadrant, the Group II (Mississippi, Alabama and two accessions from Florida) was located while Group I (Texas, Louisiana, Virginia and two accessions from Florida) was distributed at the middle portion and at the lowest part was Group III. Up to 79% of the cumulative variance was already explained by the first two components, PC1 and PC2, which accounted respectively about 72% and 7% (Figure 4.6A) of the variance. When more principal components were tested as shown in Figures 4.6B (PC1 and PC3) and 4.6C (PC1 and PC4), the PCA results did not clearly represent the three major groups detected by the dendrogram.

Result of the calculation of the eigenvalues based on the 190 individual plant samples has confirmed that the first eigenvalue was significant enough to explain the variation projected by PCA in Figure 4.6A. Calculated eigenvalues were the basis for the principal

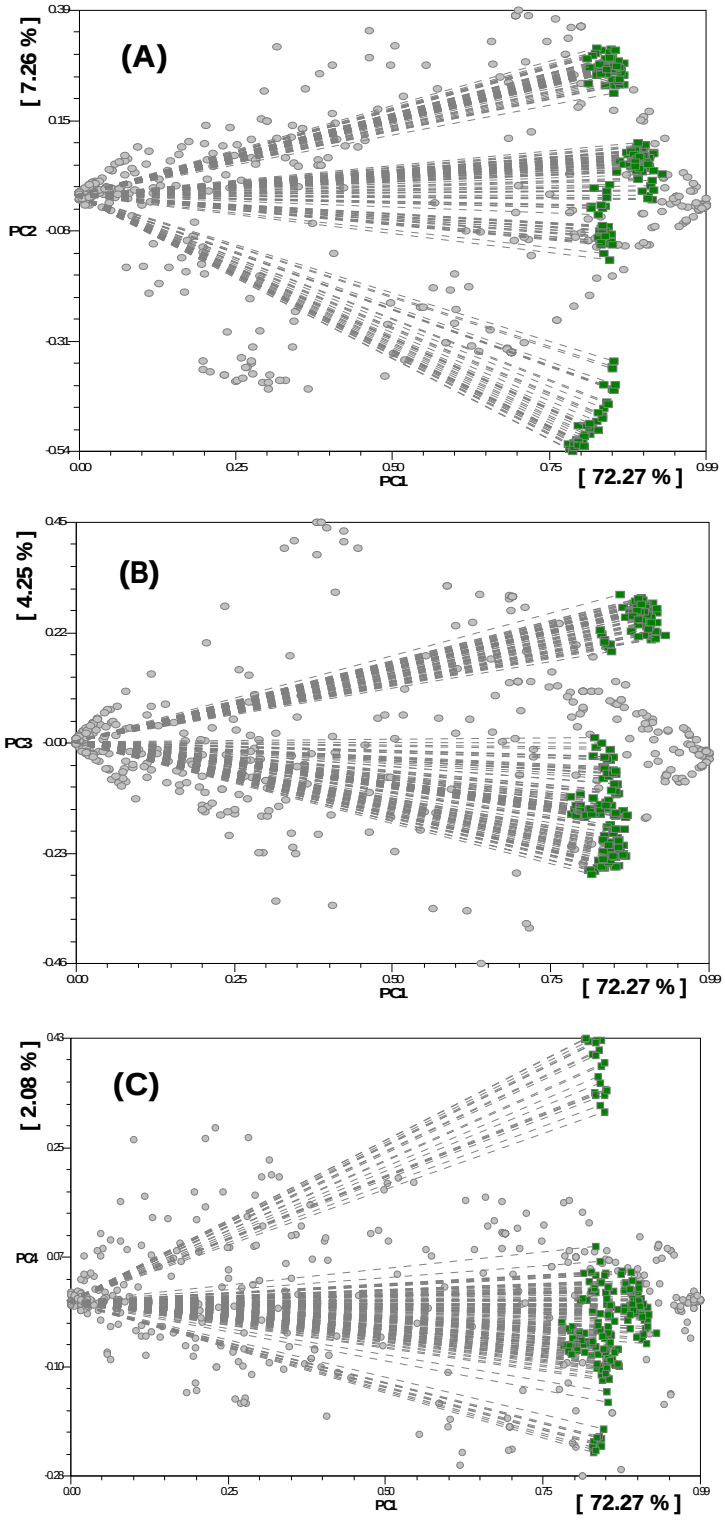


Figure 4.6 Principal component analysis of plant accessions and markers. (A) When using PC1 and PC2, 79% of variation was explained and confirmed the three groups clustered by SAHN. Additional PCA, (B) PC1 and PC3 and (C) PC1 and PC4, did not resolve the cluster obtained from SAHN.

discriminating axes in a multi-dimensional perspective, allowing spatial conformation of the SAHN groupings.

Having established the above groupings of the samples, ordination procedure was done on the same data to project the twelve primer combinations onto the principal component axes together with the plant accessions. The AFLP markers were represented in small dots while plant accessions were represented in rectangular-shapes.

Figure 4.6A indicates that at least seven primer combinations (*Eco*RI-AGG+*Mse*I-CGT, *Eco*RI-AGG+ *Mse*I-CAC, *Eco*RI-CAA+ *Mse*I-CGT, *Eco*RI-ACT+ *Mse*I-CTG, *Eco*RI-CAA+ *Mse*I-CCT, *Eco*RI-CAG+ *Mse*I-CGA and *Eco*RI-CAG+ *Mse*I-ACG) were highly correlated based on its relatively smaller vector angle from their origin. Furthermore, the proximity of these primer combinations from the grouped accessions implies its relative discriminant effect in characterizing intraspecific and interspecific similarities of the samples.

A line plot is presented in Figure 4.7 summarizing the variance proportions (%) explained by each principal component. A cumulative increase in variance (%) was expected for each addition of principal component axis, but it is less suited to clearly discriminate the actual groupings because of the difficulty involved in perceiving more than three-multidimensional structure.

4.3.4 Analysis of molecular variance

For this study, AMOVA measures within-plant variation, variation among accessions within the state, and variation among the states. The method calculates the extent of population subdivision by comparing the average heterozygosity of individual plant genetic profiles to the average heterozygosity of the total population expected under random mating

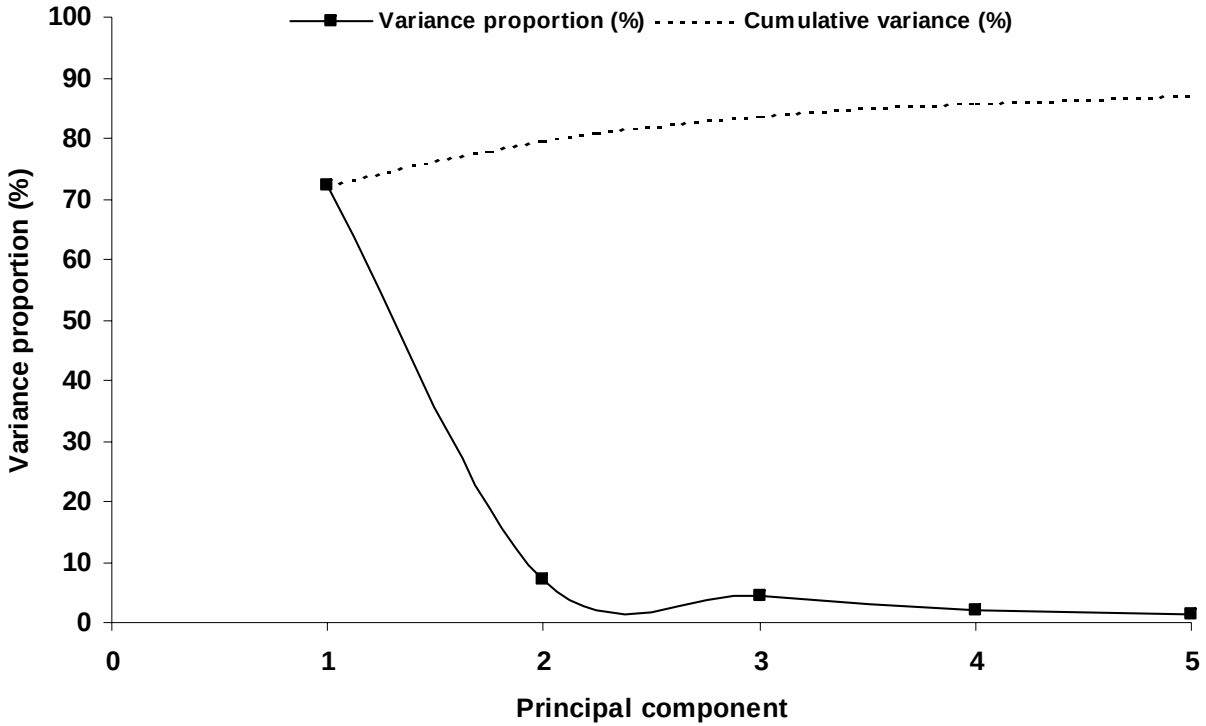


Figure 4.7 Line plot summary of variance proportions (%) explained by 5 principal components. Rate of cumulative variance (%) when more components are added.

(Wright, 1978). The estimation of *Fst*s would classify level of gene flow, *i.e.,* a low *Fst* value would suggest a high level of gene flow (Avise, 1994) and vice-versa.

AMOVA analysis from the AFLP binary matrix profile for the 190 *U. paniculata* AFLP fingerprints permitted a partitioning of the source of variations. The results of the analysis of the molecular variance after 1000 permutations showed highly significant *Fst* values for all the source of variation as presented in Table 4.7. The highest significant amount of genetic variation was observed at the state-level (*df*=6, variance component=25.3, %variation=47%; *p*=<0.001) followed by the variation among the genotype at 34% (*df*=171, variance component=18.3, *p*=<0.001). The total variation among accessions within a state was the lowest having only 19% (*df*=12, variance component=10.4, *p*=<0.001). Overall, the percentages of variation for each of the source of variation were highly significant suggesting a high level of genetic diversity.

The highest genetic variation detected at the state-level, which implies isolation by distance has confirmed the results from the UPGMA dendrogram and the PCA where the

Table 4.7 Analysis of molecular variance (AMOVA) for 190 individual plants belonging to 19 different accessions from 8 different states.

Source of variation	Degrees of freedom (d.f)	Sum of squares (SS)	Variance components	Percentage of variation	*P* values
Among states	6	4725.97	25.3	47	<0.00
Among accessions within states	12	1463.60	10.4	19	<0.00
Among genotypes	171	3127.50	18.3	34	<0.00
Total	**189**	**9317.07**	**54.0**		

accessions were grouped accordingly by its geographical origin except for those from Florida. The separation of two accessions from Florida (LA35 and LA39) can be attributed to the variation among genotypes brought about by some ecological disturbance along the state. RAPD analysis by Ranamukhaarachchi (2000a) suggested that the genetic variation observed from natural populations of *U. paniculata* in Florida was associated with frequent storm disruptions. Meanwhile, variation among accession within the state was low but statistically significant indicating substantial difference among the accessions.

The highest total variation at the state-level indicated *U. paniculata* natural populations are greatly differentiated by a wider geographic perspective. Given the apparent randomness of accession selection, some expectations were met that differentiation might exist among accessions from different states as they were separated by geographically. *Fst* values from pairwise comparisons among states indicated significant difference (p=0.05) implying isolation by distance (Excoffier et al., 1992). The number of permutation for Mantel test (Mantel, 1967) was 1000 and matrix of significance (p=0.05) for the pairwise comparison was generated after 3024 permutations.

4.3.5 Intra-population molecular diversity at the accession level and its genetic flow pattern

The significance of hierarchical *Fst* analysis from a genetic perspective is its ability to detect differentiation from a wider scale down to single-level analysis that estimates probabilities, *Fst* values and pairwise genetic distances. Consequently, the hierarchical *Fst* analysis partitioned total genetic variance into components occurring at each level of sampling, *i.e.,* among states, among accessions within states, and within plants. Differentiation of *U. paniculata* accessions by pairwise comparison and permutation tests yielded significant result at p=0.05 as confirmed by the *Fst* P values significance matrix. The

pairwise differences found from all the accessions were significantly different (p=0.05). This significant differentiation among the accessions inferred by hierarchical *Fst* analysis can be attributed to the large variations accumulated from among-genotype level thus, contributing to the highest total variation observed at the state-level.

Likewise, molecular diversity among accessions based on the marker frequencies and polymorphism was also determined by AMOVA to conclusively assess the level of genetic diversity. In Table 4.8, nucleotide diversity was categorized for each of the accession. This provides the probability that the genotypes are different based on the binary scores of "1" and "0" for each plant. It is equivalent to the gene diversity at the nucleotide level. Calculated average gene diversity over loci is computed with an assumption of no recombination and selectivity. Among the accessions, the highest was in LA47 (0.07±0.04, Petit Bois Is., Mississippi), LA33 (0.07±0.04, Henderson Beach, Florida), LA15 (0.07±0.04, Fourchon Beach, Louisiana) and LA41 (0.07±0.04, West Ship Is., Mississippi) (Figure 4.8). Meanwhile, the average gene diversity per loci values ranged from as low as 0.02±0.01 to as high as 0.07±0.04. The number of polymorphic nucleotide sites for each accession was highest in LA47 (n=139, Petit Bois Is., Mississippi) followed by the accessions from Florida, LA39 (n=135, Perdido Key) and LA33 (n=134, West Crooked Is.). The lowest was observed in LA53 (n=41, Assateague Is., Virginia).

In Figure 4.9, wide ranges of polymorphic nucleotide sites were detected in Texas accession (88-137), Florida (96-135) and South Carolina (55-76) while Louisiana, Mississippi, Alabama and North Carolina have fairly small range of nucleotide polymorphism. With the molecular variations among accessions being significantly different, the level of genetic flow of *U. paniculata* can be indirectly postulated.

Table 4.8 Molecular diversity indices using distance method to analyze intra-population level of polymorphism.

Accessions	State	Sample size	Number of usable nucleotide sites	Number of polymorphic sites	Mean number of pairwise difference	Average gene diversity over loci
LA2	TX	10	693	88	34.4±16.4	0.05±0.03
LA5	TX	10	693	91	32.8±15.6	0.05±0.02
LA9	TX	10	693	107	37.7±17.9	0.05±0.02
LA17	TX	10	693	131	45.4±21.6	0.07±0.03
LA15	LA	10	693	130	48.0±22.8	0.07±0.04
LA16	LA	10	693	112	42.8±20.4	0.06±0.03
LA41	MS	10	693	128	47.8±22.6	0.07±0.04
LA47	MS	10	693	139	49.9±23.7	0.07±0.04
LA19	AL	10	693	113	41.6±19.8	0.06±0.03
LA21	AL	10	693	107	38.7±18.4	0.05±0.03
LA29	FL	10	693	115	39.8±18.9	0.06±0.03
LA33	FL	10	693	134	50.5±23.9	0.07±0.04
LA35	FL	10	693	96	38.5±18.3	0.05±0.03
LA39	FL	10	693	135	46.2±21.9	0.07±0.03
NC15	SC	10	693	55	19.9± 9.6	0.03±0.01
NC19	SC	10	693	76	28.7±17.7	0.04±0.02
NC1	NC	10	693	53	19.5± 9.4	0.03±0.01
NC11	NC	10	693	59	18.8± 9.1	0.03±0.01
LA53	VA	10	693	41	13.7± 6.7	0.02±0.01

Marker frequencies among individual genotype was tested with Markov chain random walk algorithm (number of Markov chain steps = 10000; dememorisation steps = 1000; p=0.05) and correlated distance matrices set at 1000 number of permutations for significance testing. Results from the measurement of the extent of population subdivision by comparing the average heterozygosity of individual plant to the average heterozygosity of the total populations expected under random mating (Wright, 1978) computed very low *Fst* values from 0.0-0.00033. These *Fst* values used as an indirect estimate of gene flow (Slatkin,

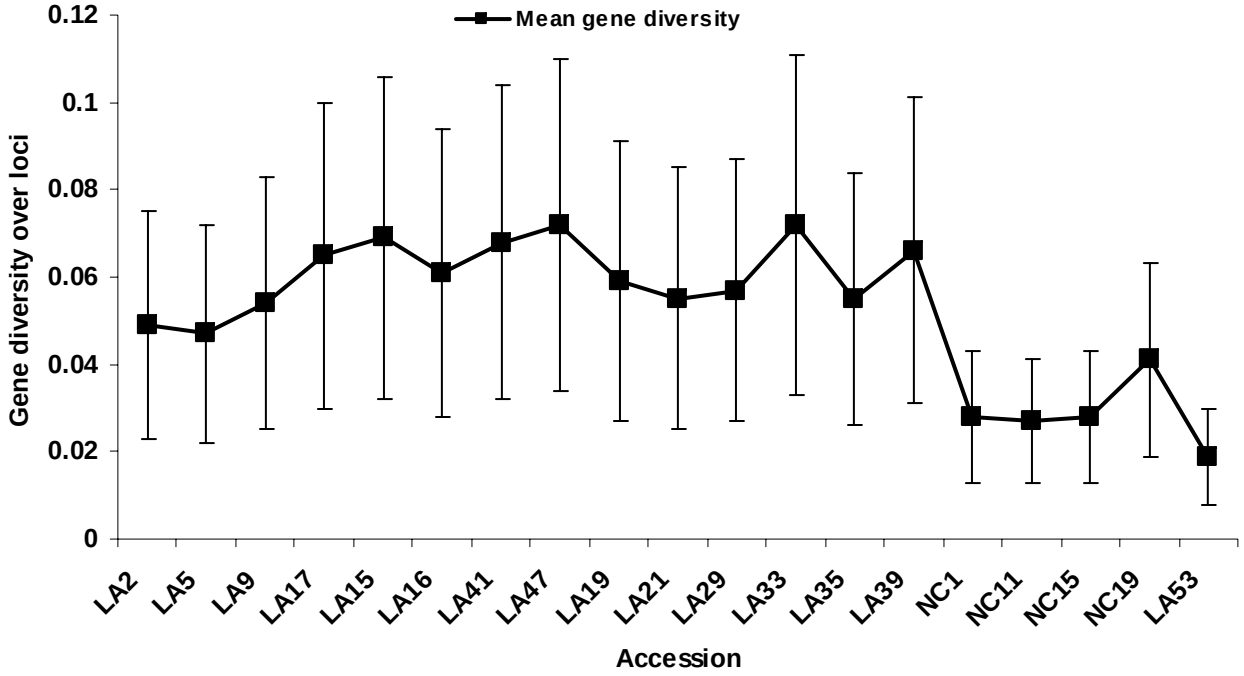


Figure 4.8 Intra-population molecular diversity analysis showing the mean genetic diversity by accession (± SE). All accessions have significant difference at $p = 0.05$.

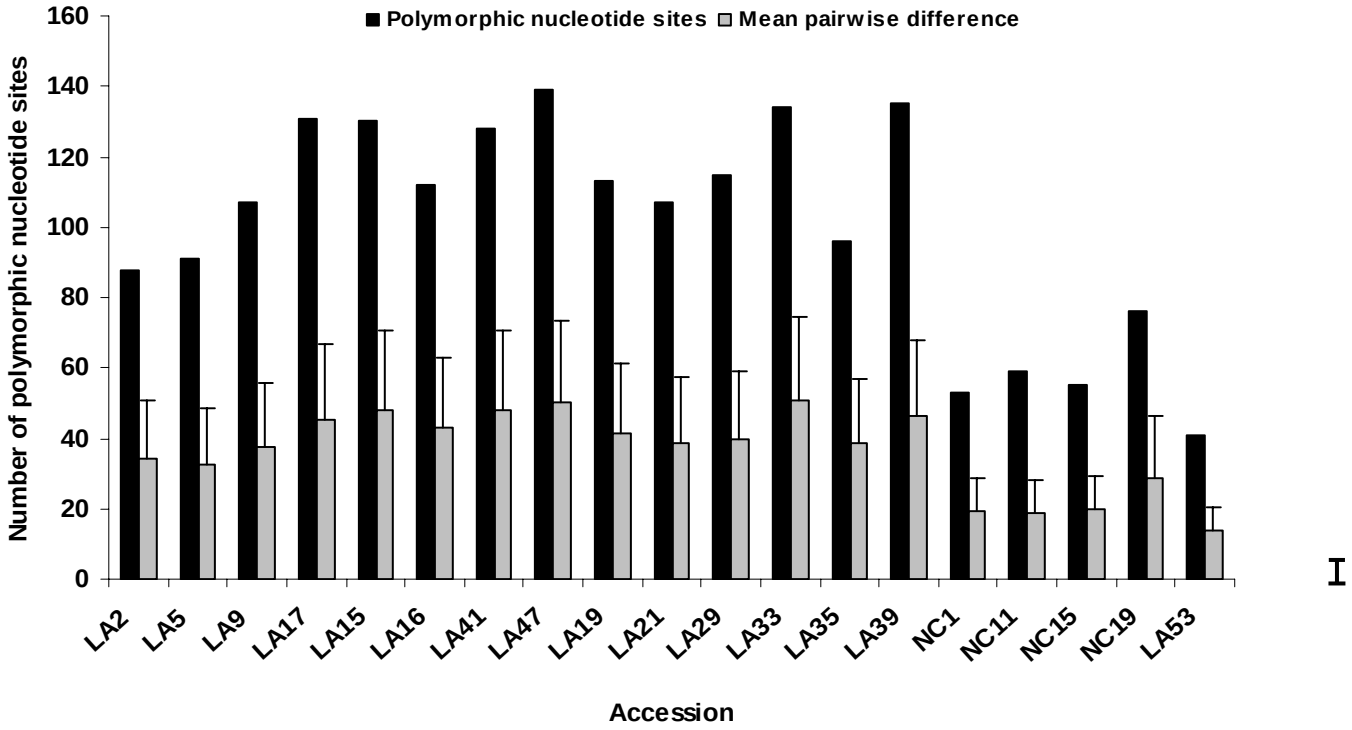


Figure 4.9 Intra-population molecular diversity analyses showing the total number of polymorphic nucleotides and the pairwise difference by accession (± SE). All accessions have significant difference at p = 0.05.

1985) indicated a high level of gene flow (Avise, 1994) among the studied accessions of *U. paniculata*. This high level of gene flow can be attributed to the amount of genetic differences observed between the accessions as resolved by the AFLP marker polymorphism.

Gene flow level assumption in this study refers to the genetic variability at marker loci as described by Slatkin and Barton (1989). Under a stringent condition such as utilizing AFLP, the state of the population genetic variability was measured and the estimate of gene flow was obtained from the allele frequencies in the studied natural populations (Mallet, 2000). As further described by Mallet (2000), the gene flow estimation referred to, in this case, can be best explained from the assumption that a pair of populations differs in gene frequency at each loci and the exchange of individuals ensures gametic correlations. So that when the amount of genetic difference increases, the effect of gene flow also increases. Result from the computation of pairwise differences between accessions is presented in Table 4.9.

The calculated pairwise difference were all significantly different at p=0.05. This was calculated by counting the number of different alleles between genotypes using the Kronecker function equal to 1 if the alleles of the i-th locus are identical for both genotypes and equal to 0 if otherwise. In estimating genetic structure indices, this choice amounts to estimating weighted *Fst* statistics over all loci (Excoffier and Quattro, 1992). Each accession being significantly different has genetic variation values ranging from as low as 0.12 (between NC1 and NC11) to a high of 0.85 (between NC1 and NC15 and LA53, Virginia). The lowest ranges of genetic variation observed within state were those from Texas (0.15-0.29) and Louisiana (0.13-0.28). Other accessions have larger range of variations.

Table 4.9 Pairwise *Fst* comparison differences among accessions at *p*=0.05.

LA	2	5	9	17	15	16	53	41	47	19	21	29	33	35	39	1	11	15	19
2	0.00																		
5	0.15	0.00																	
9	0.29	0.21	0.00																
17	0.24	0.19	0.61	0.00															
15	0.27	0.23	0.23	0.14	0.00														
16	0.34	0.28	0.33	0.26	0.13	0.00													
53	0.69	0.71	0.72	0.66	0.66	0.66	0.00												
41	0.56	0.54	0.54	0.51	0.51	0.53	0.71	0.00											
47	0.57	0.56	0.55	0.53	0.52	0.54	0.72	0.14	0.00										
19	0.61	0.59	0.59	0.56	0.56	0.58	0.76	0.21	0.13	0.00									
21	0.62	0.61	0.62	0.58	0.58	0.59	0.78	0.26	0.22	0.16	0.00								
29	0.66	0.65	0.66	0.61	0.61	0.62	0.78	0.38	0.35	0.35	0.26	0.00							
33	0.59	0.59	0.60	0.56	0.56	0.57	0.74	0.31	0.27	0.26	0.26	0.19	0.00						
35	0.64	0.64	0.62	0.59	0.58	0.61	0.79	0.61	0.61	0.64	0.64	0.64	0.56	0.00					
39	0.62	0.61	0.60	0.58	0.56	0.60	0.76	0.59	0.59	0.61	0.62	0.62	0.55	0.36	0.00				
1	0.77	0.79	0.78	0.75	0.75	0.76	0.85	0.76	0.76	0.78	0.80	0.80	0.76	0.77	0.69	0.00			
11	0.77	0.78	0.78	0.75	0.74	0.76	0.85	0.76	0.76	0.78	0.79	0.80	0.76	0.76	0.69	0.12	0.00		
15	0.74	0.77	076	0.72	0.72	0.74	0.84	0.74	0.73	0.76	0.78	0.78	0.74	0.74	0.67	0.50	0.46	0.00	
19	0.69	0.71	0.72	0.68	0.67	0.69	0.80	0.69	0.69	0.72	0.73	0.75	0.70	0.70	0.62	0.52	0.49	0.15	0.00

4.3.6 Genetic variation at the state-level

A second AMOVA analysis was done to further validate the observed significant variation at the state-level. By pooling all the genotypes from each state, the analysis has confirmed the significant differences among the states.

Table 4.10 shows that the total variation at the state-level accounted to 54%. This result reveals how the significant variation found among the different states can also be attributed to the significant variation found from among genotypes.

Table 4.10 Analysis of molecular variance (AMOVA) for 190 individual plants belongs to 19 different accessions from 8 different states.

Source of variation	Degrees of freedom (d.f)	Sum of squares (SS)	Variance components	Percentage of variation	*P* values
Among states	6	4725.968	28.9	54	<0.00
Among genotypes	183	4591.100	25.1	46	<0.00
Total	189	9317.068	54.0		

The *Fst* P values matrix of significance confirmed these differences among states. The pairwise state-level comparisons was statistically different at p=0.05.

Table 4.11 shows the pairwise differences of each state. The genetic variation ranged from as low as 0.14 (between Mississippi and Alabama) to a high of 0.84 (between Virginia and North Carolina).

4.3.7 Cluster analysis as influenced by percent polymorphism

As presented in Table 4.6, the total number of AFLP bands produced among the primer combinations differ from each other. Accordingly, the percent polymorphism also depends on the number of polymorphic bands. Irrespective of the number of polymorphic bands, cluster analyses were made to analyze those primer combinations that generated <50% polymorphism and >70% polymorphism.

Table 4.11 Population pairwise *Fst*s comparison differences among states at *p*=0.05.

	Texas	Louisiana	Mississippi	Alabama	Florida	South Carolina	North Carolina	Virginia
Texas	0.00							
Louisiana	0.17	0.00						
Mississippi	0.49	0.48	0.00					
Alabama	0.54	0.54	0.14	0.00				
Florida	0.40	0.38	0.26	0.24	0.00			
South Carolina	0.66	0.69	0.69	0.73	0.51	0.00		
North Carolina	0.72	0.74	0.74	0.77	0.57	0.45	0.00	
Virginia	0.58	0.59	0.66	0.73	0.53	0.79	0.84	0.00

Figure 4.10 shows the results of the UPGMA clustering when using the above criteria. When three primer combinations (*Eco*RI-AGG+*Mse*I-CAC, *Eco*RI-CAG+*Mse*I-CCT and *Eco*RI-CAA+*Mse*I-CCT) having < 50% polymorphism were used to generate a dendrogram, the clustering of genetically similar accession was found out to be inconclusive and was not orderly discriminated (Figure 4.10A).

A large difference was observed when the same numbers of primer combinations (*Eco*RI-ACT+*Mse*I-CTC, *Eco*RI-CAG+*Mse*I-ACG and *Eco*RI-CAG+*Mse*I-CGA) having > 70% polymorphism were tested.

Figure 4.10B presents dendrogram similar to what has been established in Figure 4.3 when all twelve-primer combinations were utilized. This observation implies the importance in choosing AFLP primer combinations. While ensuring that a higher level of polymorphism can be detected by a certain primer combination, one could minimize the number of primer combinations to use for the analysis.

4.3.8 Genetic similarity of individual plants characterized by the 12 primer combinations

The dendrograms presented in Figures 4.11-4.18 were collectively reflected in Figure

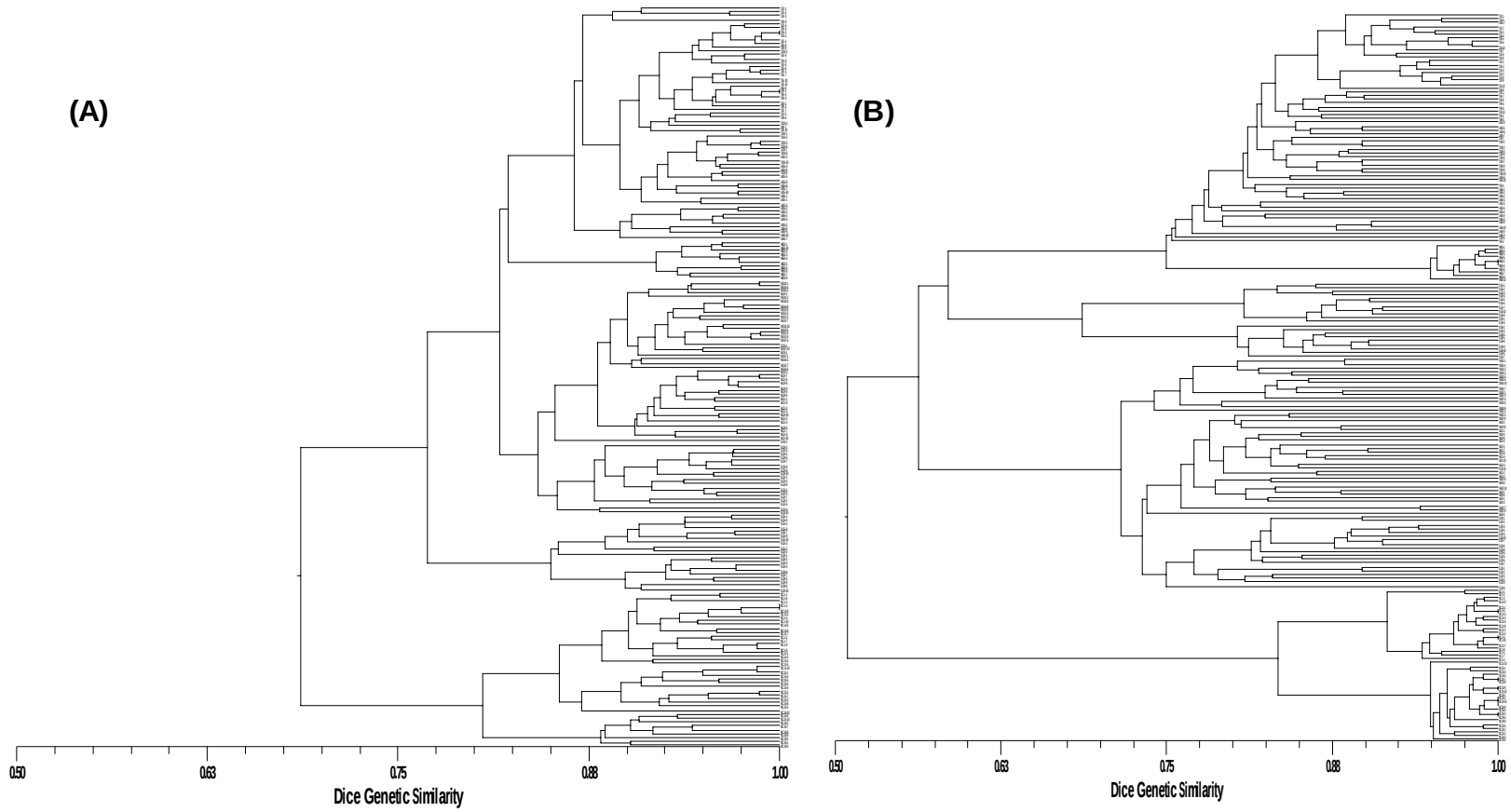


Figure 4.10 UPGMA dendrogram results as affected by the percentage of polymorphisms using the same number of primer combinations (3 *Eco*RI+*Mse*I). Similar Dice coefficient was used for all 190 *Uniola paniculata* L. plants belonging to 19 accessions collected from 8 states: Texas, Louisiana, Mississippi, Alabama, Florida, North Carolina and Virginia. (A) Cluster result when using 3 primer combinations with <50% polymorphism rate. (B) Cluster result when using 3 primer combinations with >70% polymorphism rate.

4.3. For a detailed representation of the genetic similarity of individual plant from each accession for every state, UPGMA dendrograms were produced utilizing the same 417 polymorphic bands resolved from the twelve primer combinations.

Results from the cluster analyses by state have shown that the plants from Texas (LA2, LA5, LA9 and LA17) have genetic similarity values ranging from 86% to 95% (Figure 4.11). Plants from the two accessions (LA2 and LA5) were mixed and joined at 0.88. LA9 and LA17 plants were accordingly ordered by its accession ID and joined at 0.88 except for one plant (LA17-5).

Genetic similarity among Louisiana plants (Figure 4.12) ranged from 85% to 93% and individual plants were grouped accordingly to its accession ID. In Mississippi, genetic similarity ranged from 82% to 91% (Figure 4.13) while Alabama similarity ranged from 84% to 93% (Figure 4.14). Florida plants have the highest observed variation with a similarity coefficient ranging from 69% to 94% (Figure 4.15). South Carolina (Figure 4.16) and North Carolina (Figure 4.17) have a range of similarity from 90-98% while Virginia (Figure 4.18) plants were highly similar with similarity ranging from 95-98%, respectively.

4.3.9 AFLP fingerprints of individual plants as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination

AFLP profiles of individual *U. paniculata* were presented in Figures 4.19-4.25. Fingerprints of each plant from each accession were generated by only one primer combination, *Eco*RI-CAG+*Mse*I-CGA, which generated the highest polymorphism. The dendrograms attached to each plant fingerprint do not represent the overall genetic structure of the plant populations. The cluster analyses were done on all bands, which include both the polymorphic and monomorphic. The fingerprints, in this case, were provided for plants'

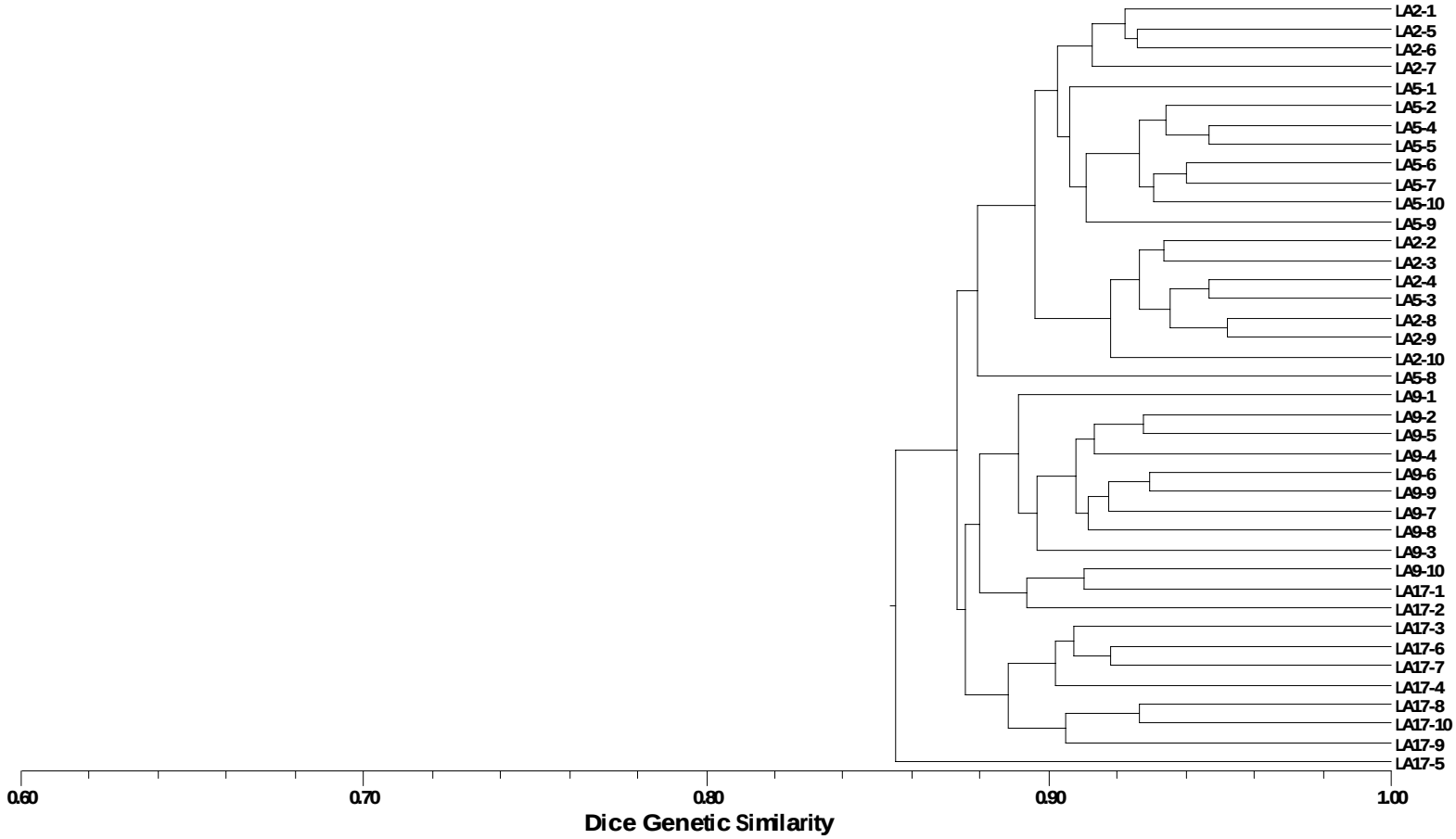


Figure 4.11 Genetic similarity of individual plants belonging to 4 Texas accessions (LA2, LA5, LA9 and LA17) based on 417 polymorphic bands resolved by the 12 primer combinations.

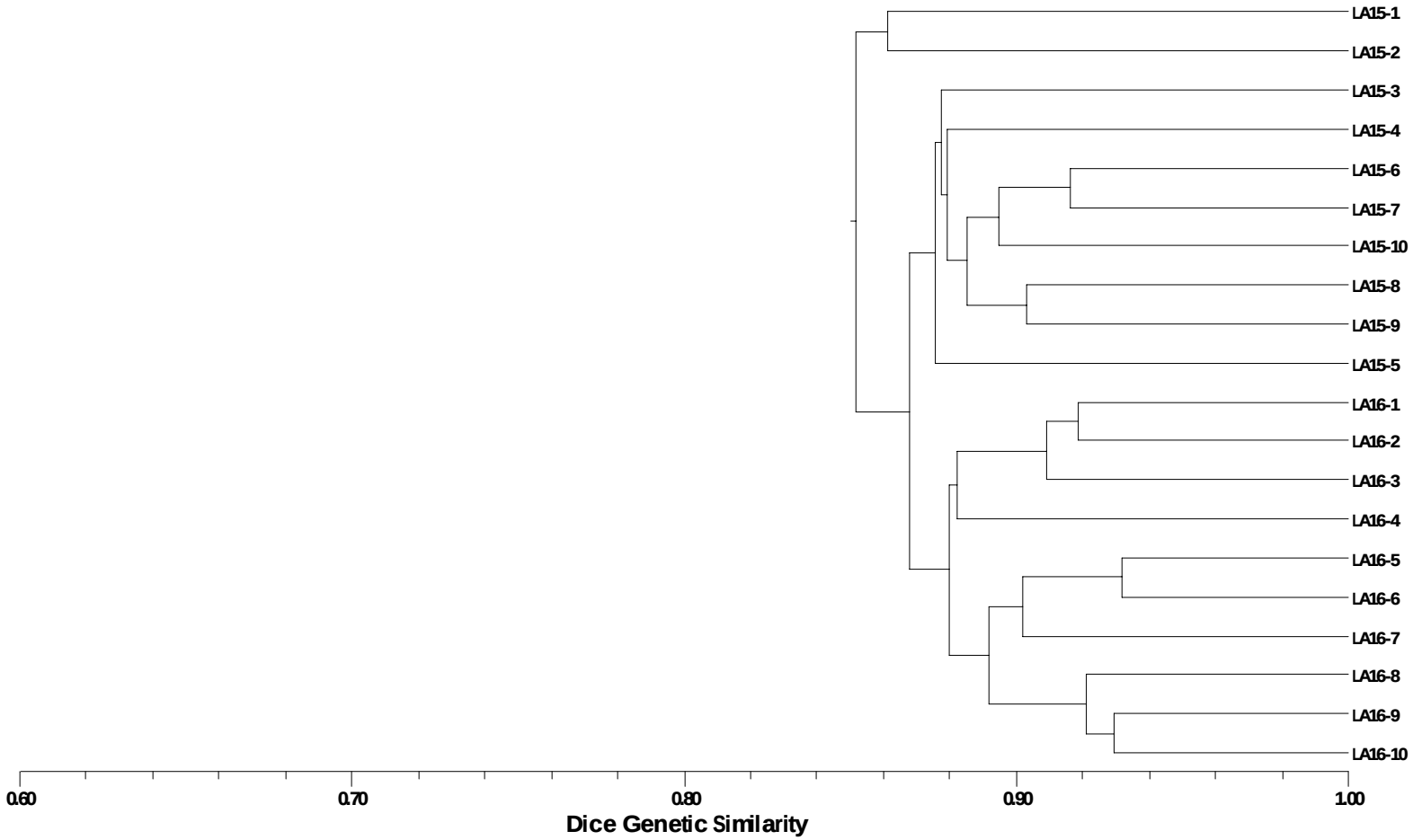


Figure 4.12 Genetic similarity of individual plants belonging to 2 Louisiana accessions (LA15 and LA16) based on 417 polymorphic bands resolved by the 12 primer combinations.

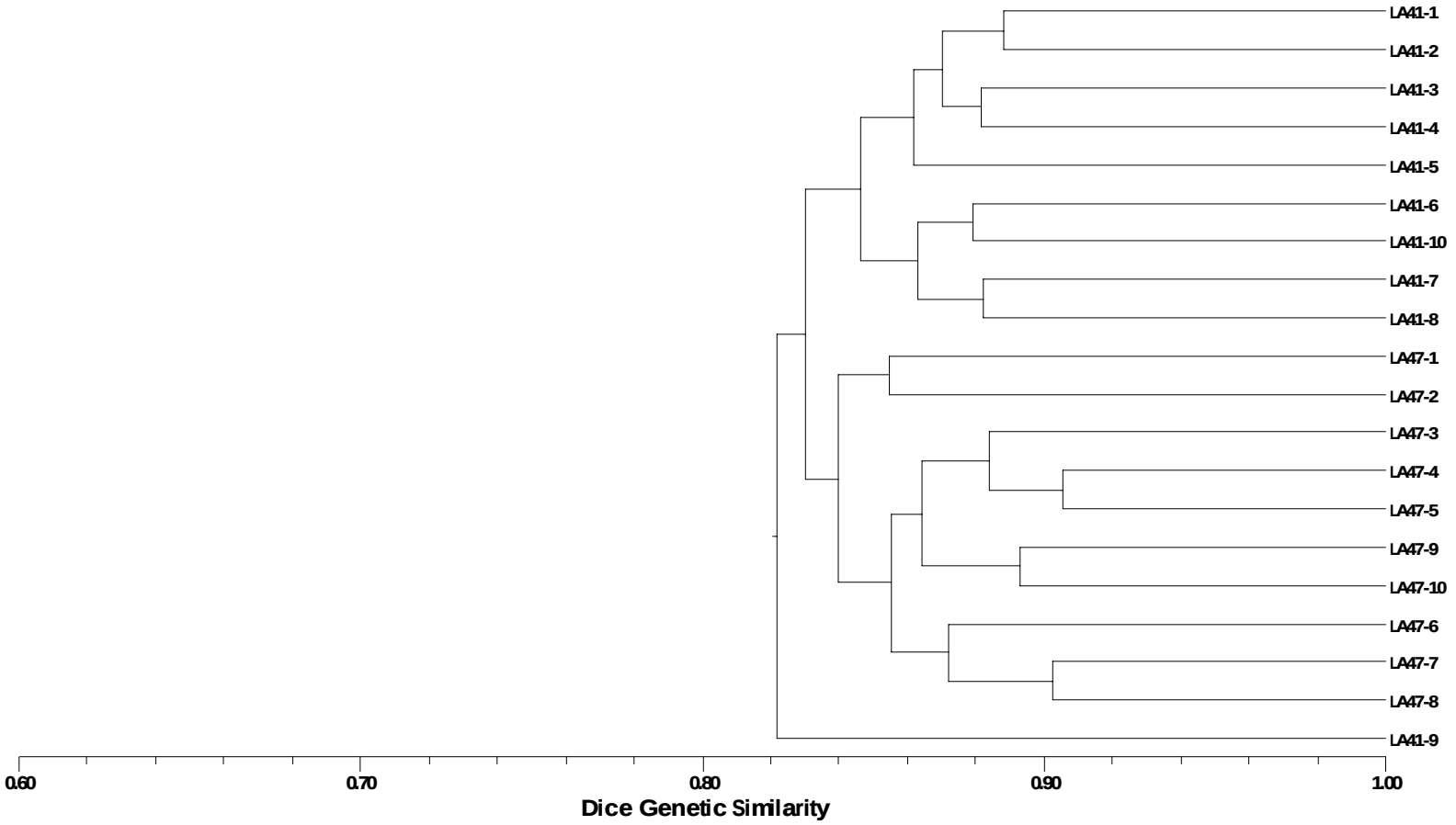


Figure 4.13 Genetic similarity of individual plants belonging to 2 Mississippi accessions (LA41 and LA47) based on 417 polymorphic bands resolved by the 12 primer combinations

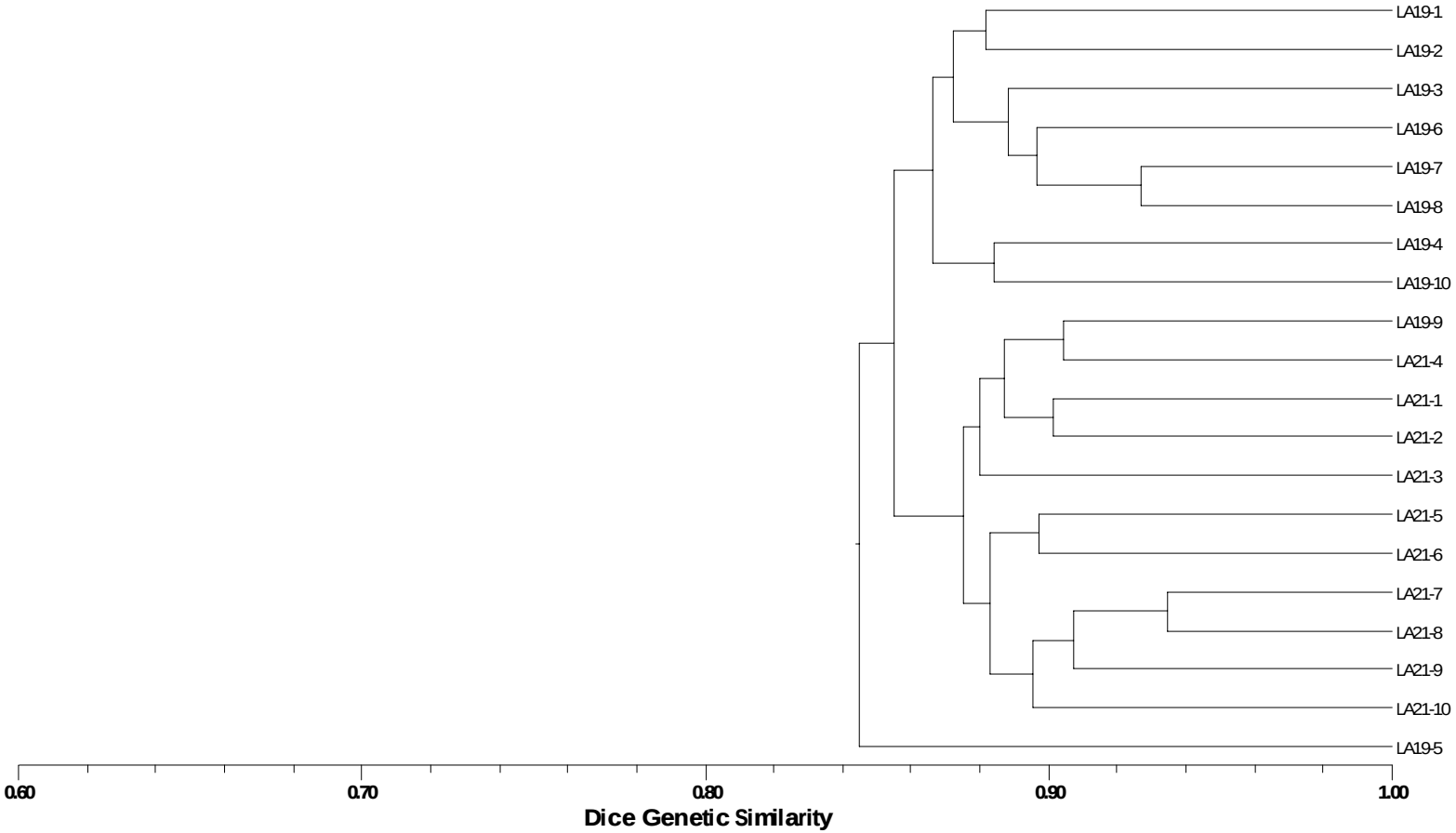


Figure 4.14 Genetic similarity of individual plants belonging to 2 Alabama accessions (LA19 and LA21) based on 417 polymorphic bands resolved by the 12 primer combinations.

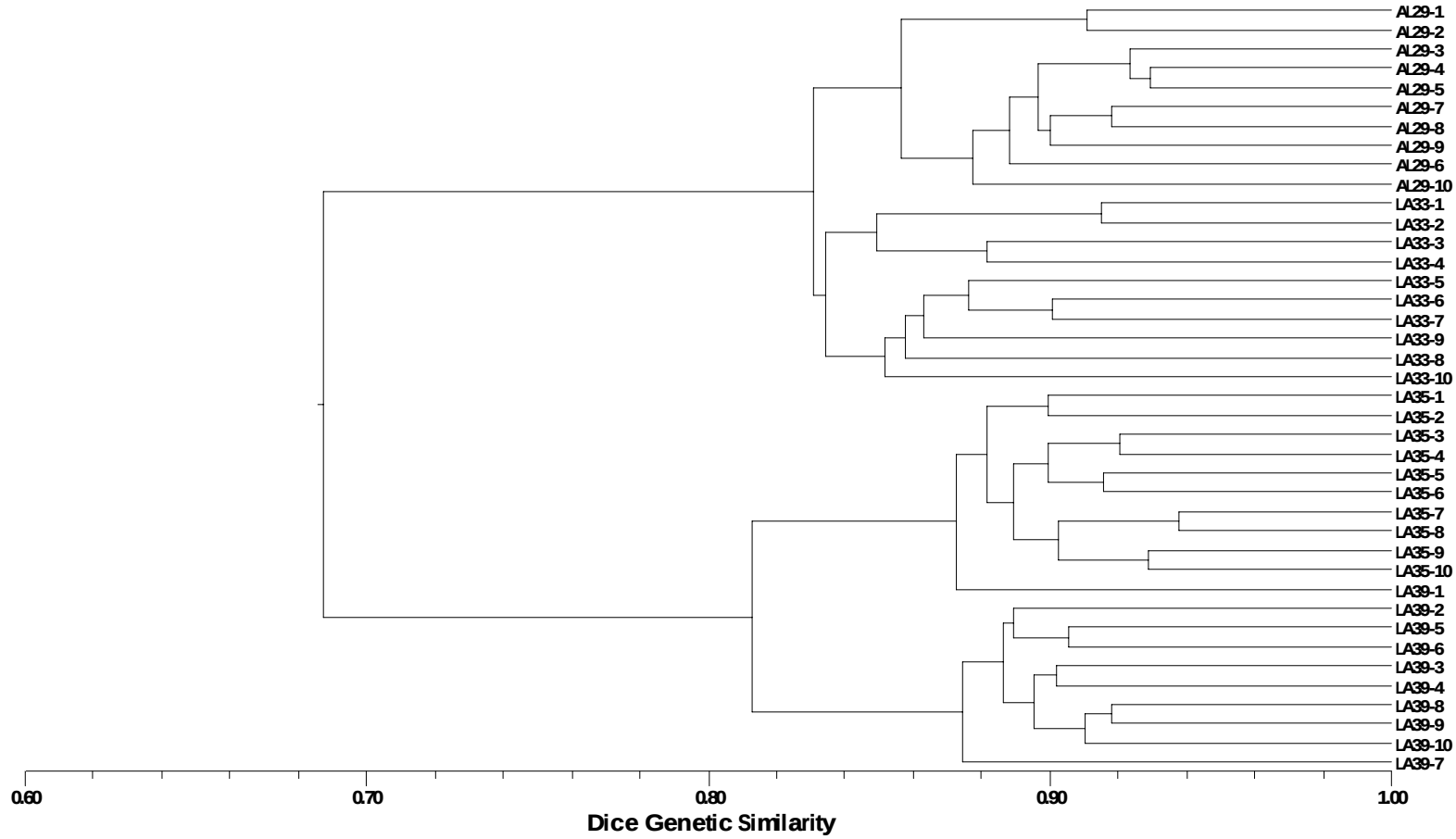


Figure 4.15 Genetic similarity of individual plants belonging to 2 Florida accessions (LA29, LA33, LA35 and LA39) based on 417 polymorphic bands resolved by the 12 primer combinations.

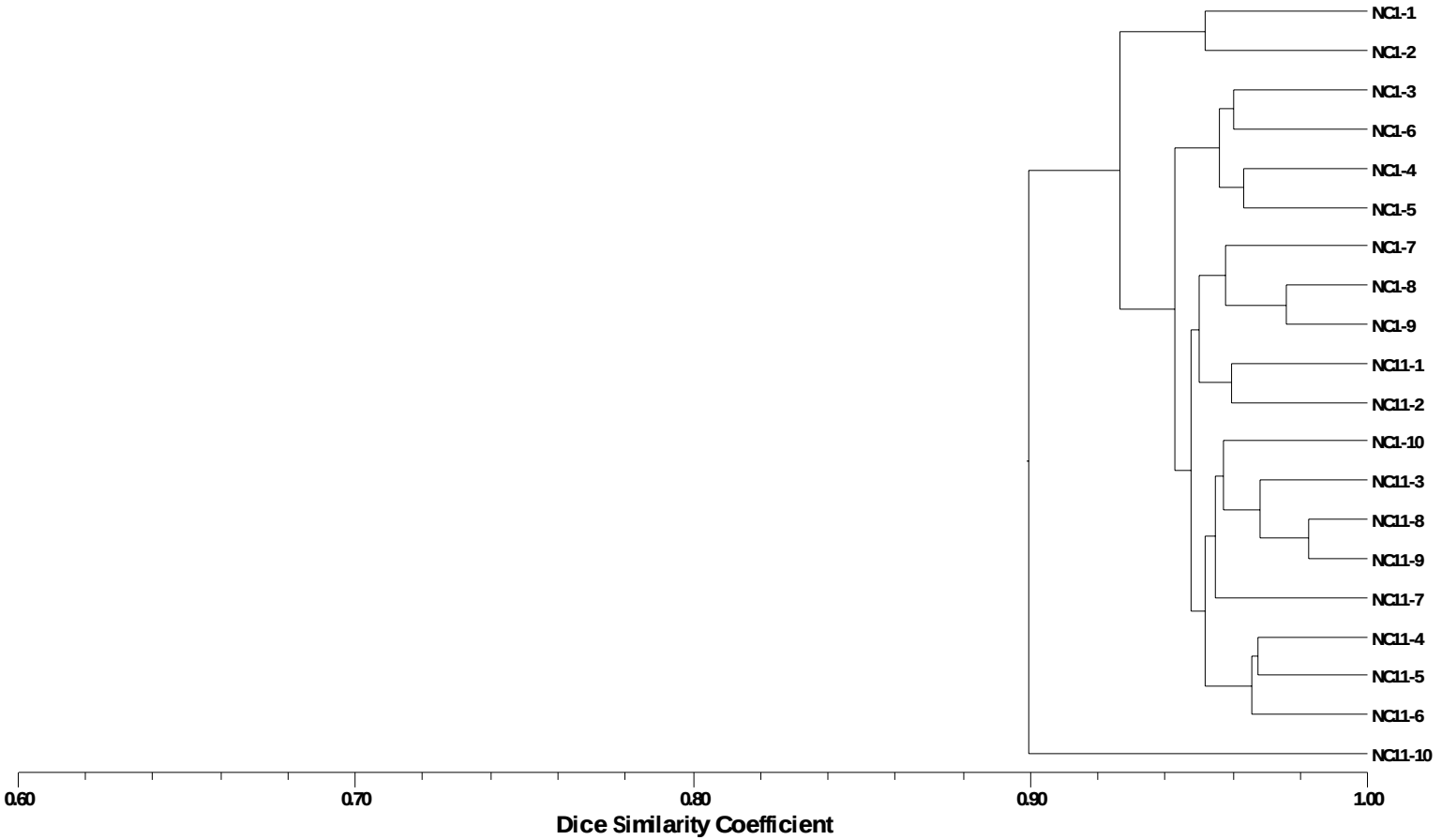


Figure 4.16 Genetic similarity of individual plants belonging to 2 South Carolina accessions (NC15 and NC19) based on 417 polymorphic bands resolved by the 12 primer combinations.

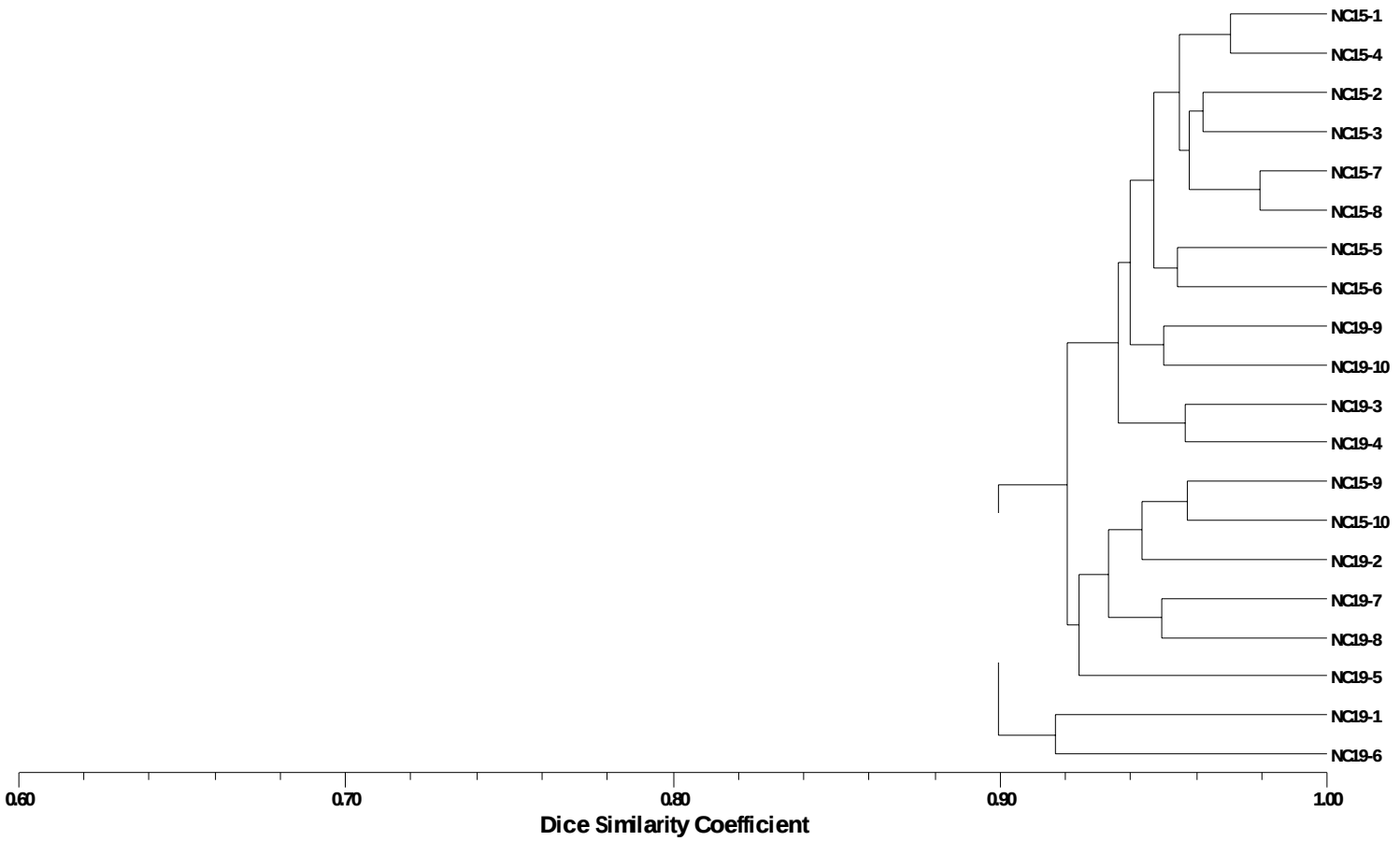


Figure 4.17 Genetic similarity of individual plants belonging to 2 North Carolina accessions (NC1 and NC11) based on 417 polymorphic bands resolved by the 12 primer combinations.

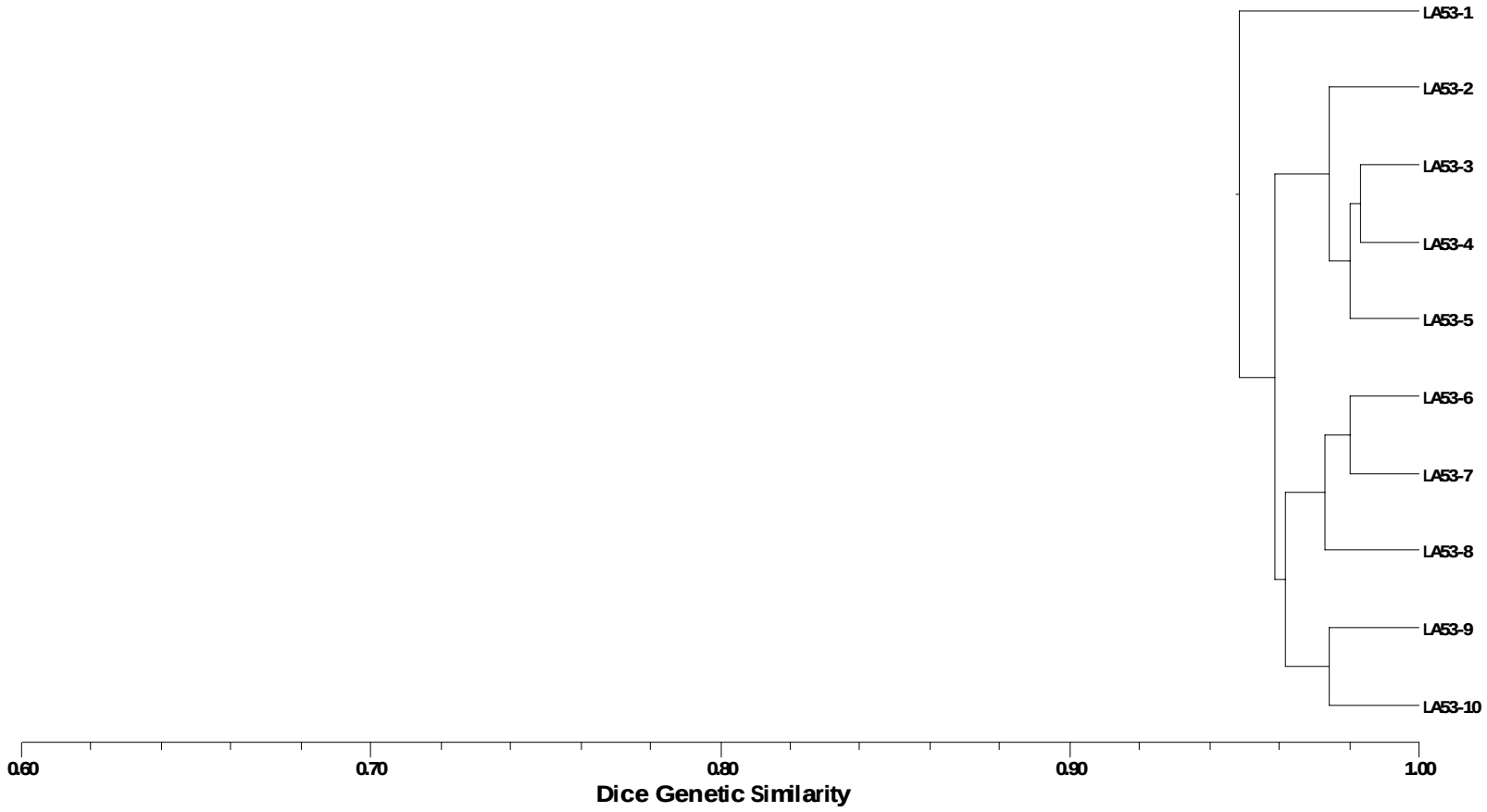


Figure 4.18 Genetic similarity of individual plants belonging to one Virginia accession (LA53) as revealed by the 417 polymorphic bands resolved by the 12 primer combinations.

identification purposes based on the AFLP profile generated by *Eco*RI-CAG+*Mse*I-CGA combination. In a wider perspective, this provides sufficient discriminatory ability to allow effective varietal identification based on the AFLP fingerprints.

When using *Eco*RI-CAG+*Mse*I-CGA alone, the genetic similarity of Texas plants (Figure 4.19) ranged from 69-100%, *i.e.,* a polymorphic and monomorphic band was included in the analyses. Likewise, plants from South Carolina (Figure 4.24), North Carolina (Figure 4.25), Louisiana (Figure 4.20) and Virginia (Figure 4.26) accessions have similarity coefficients as high as 100% with ranges from as low as 59-100%, 66-100%, and 92-100%, respectively. Genetic similarity in Mississippi (Figure 4.21) was from 64-86%. Alabama (Figure 4.22) has a range of 68-86% and Florida (Figure 4.23) has 66-95%.

4.3.10 Distinct AFLP bands and primer combinations

UPGMA dendograms produced by each primer combination have shown obvious differences. Each primer combination distinctly resolves different clustering patterns and only provides unclear groupings of plants within an accession. This is expected because each primer combination sampled different regions of the genome. Thus, molecular characterization requires substantial number of polymorphic primer combinations that generates markers from all over the genome. In this study, it is established that 3 primer combinations (*Eco*RI-CAG+*Mse*I-CGA, *Eco*RI-ACT+*Mse*I-CTC and *Eco*RI-CAG+*Mse*I-ACG) can be used to substantially resolve the genetic structure of *U. paniculata*.

Interestingly, unique AFLP bands are observed in ten accessions (LA2, LA9, LA17, LA15, LA41, LA47, LA35, LA39, NC1 and NC15) from six states (Texas, Louisiana, Mississippi, Florida, South Carolina and North Carolina). These AFLP bands are obviously distinct from the rest of the accessions. This certain band may probably link to novel gene or traits inherent to the accession or geographic location.

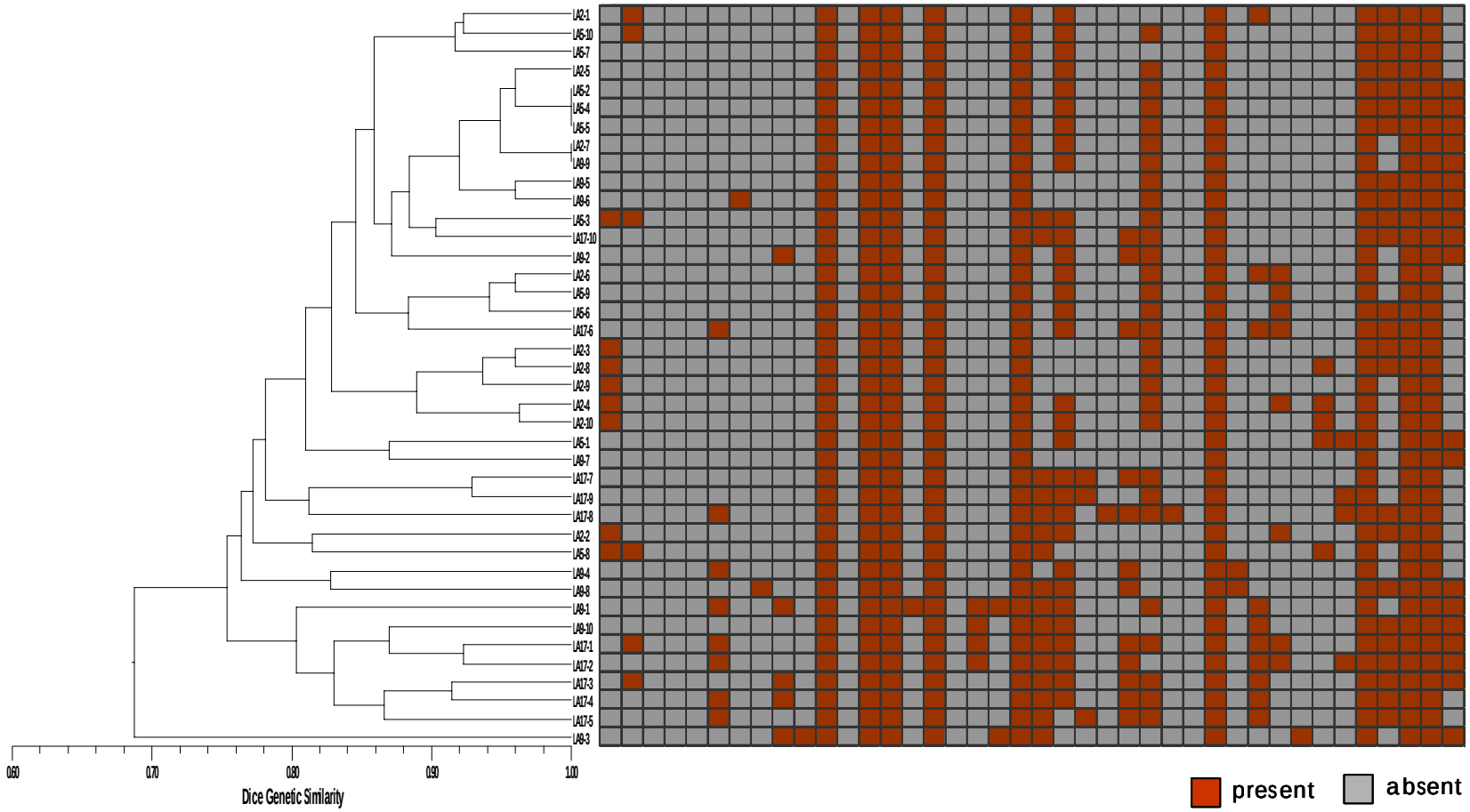


Figure 4.19 Genetic similarity and AFLP fingerprints of individual plants belonging to 4 Texas accessions (LA2, LA5, LA9 and LA17) as revealed by EcoRI-CAG+MseI-CGA primer combination.

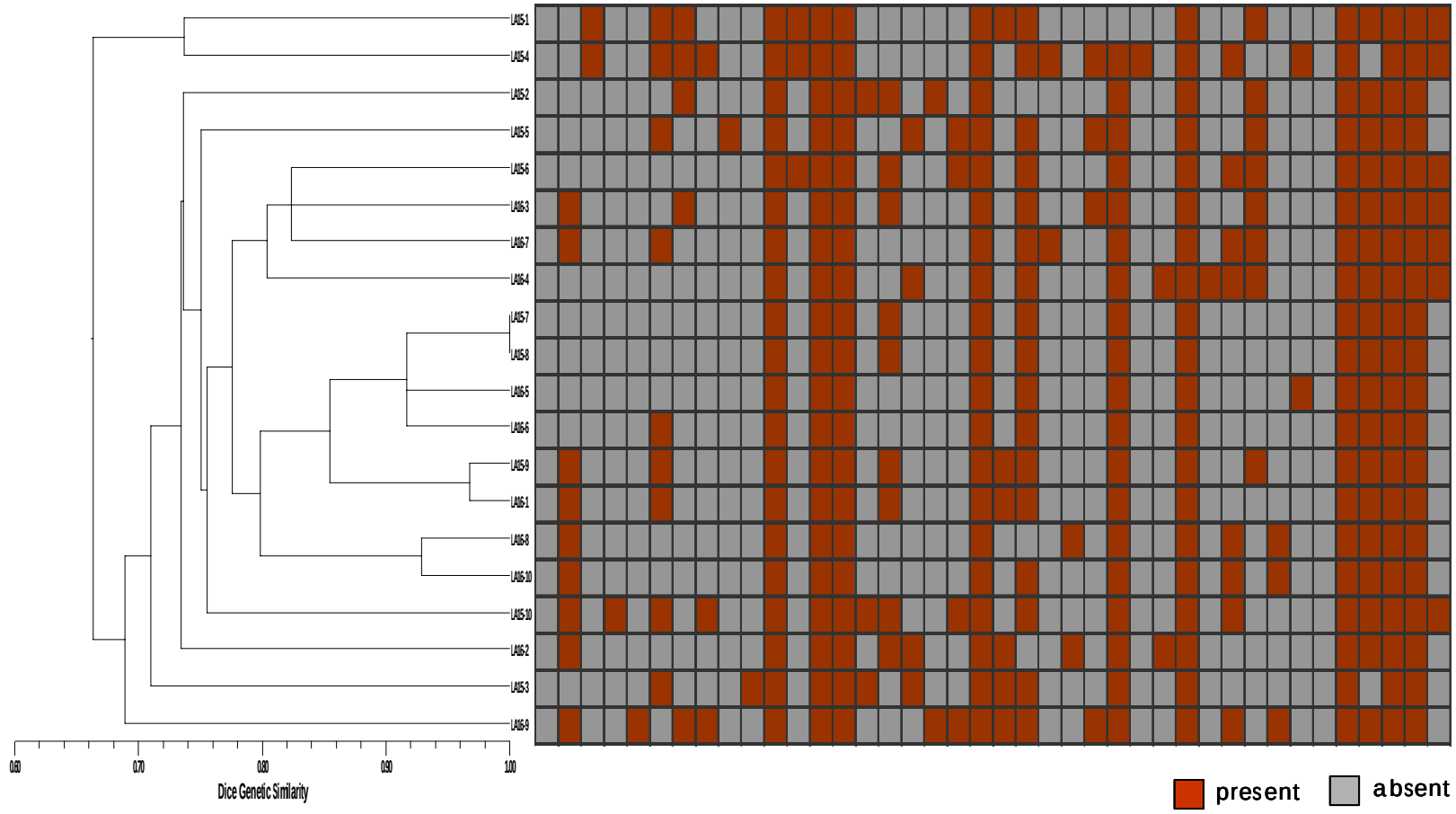


Figure 4.20 Genetic similarity and AFLP fingerprints of individual plants belonging to 2 Louisiana accessions (LA15 and LA16) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

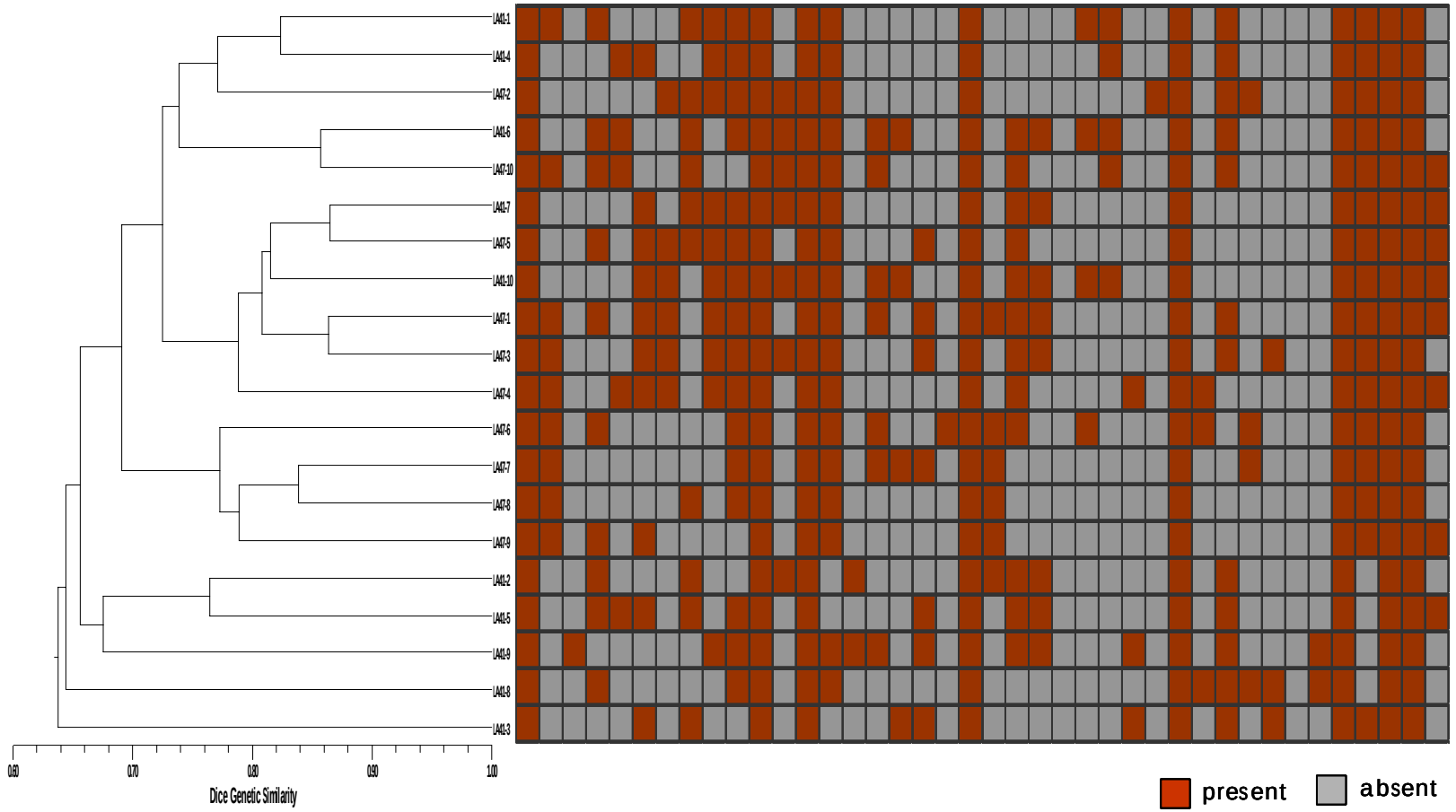


Figure 4.21 Genetic similarity and AFLP fingerprints of individual plants belonging to 2 Mississippi accessions (LA41 and LA47) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

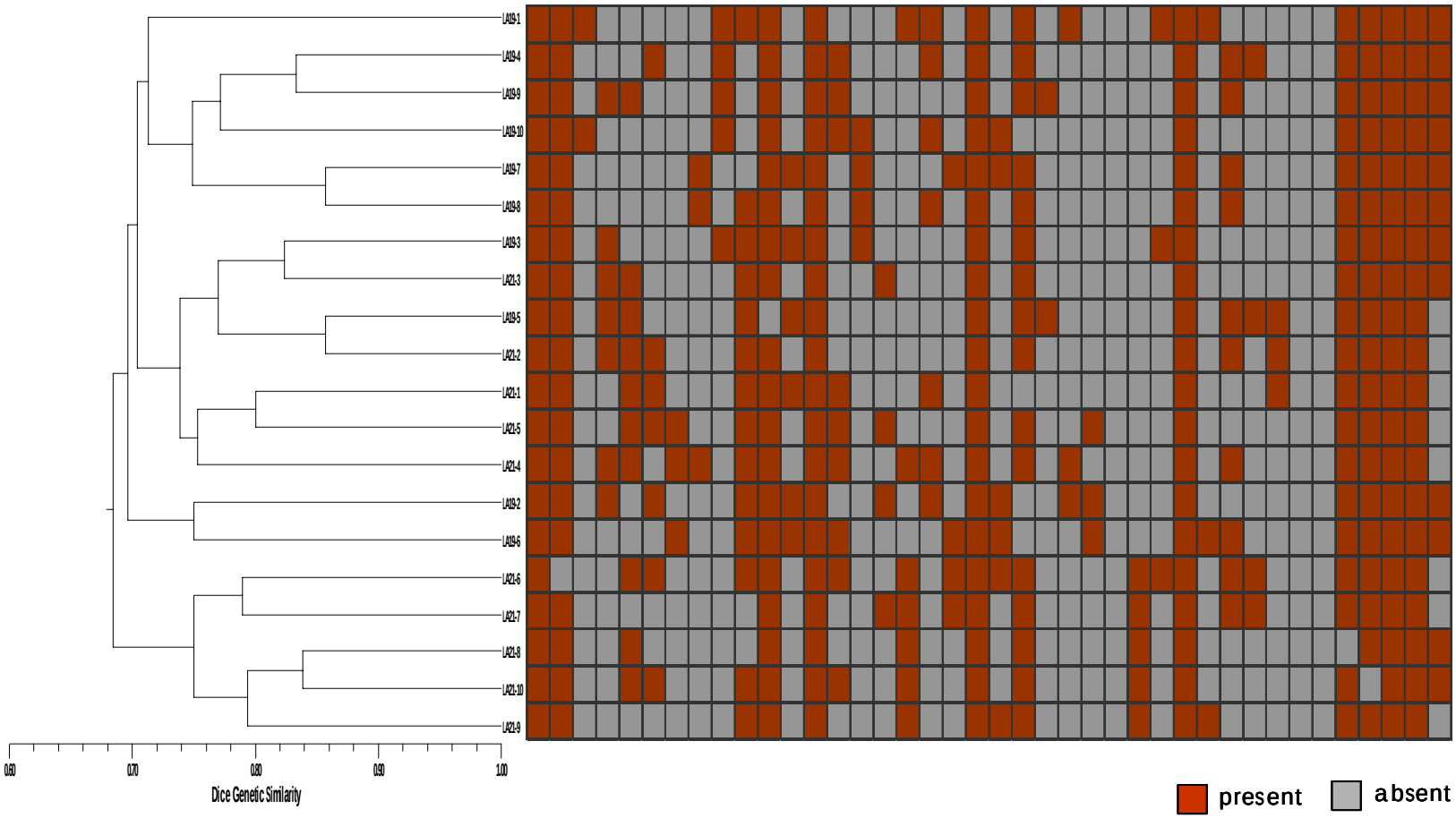


Figure 4.22 Genetic similarity and AFLP fingerprints of individual plants belonging to 2 Alabama accessions (LA19 and LA21) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

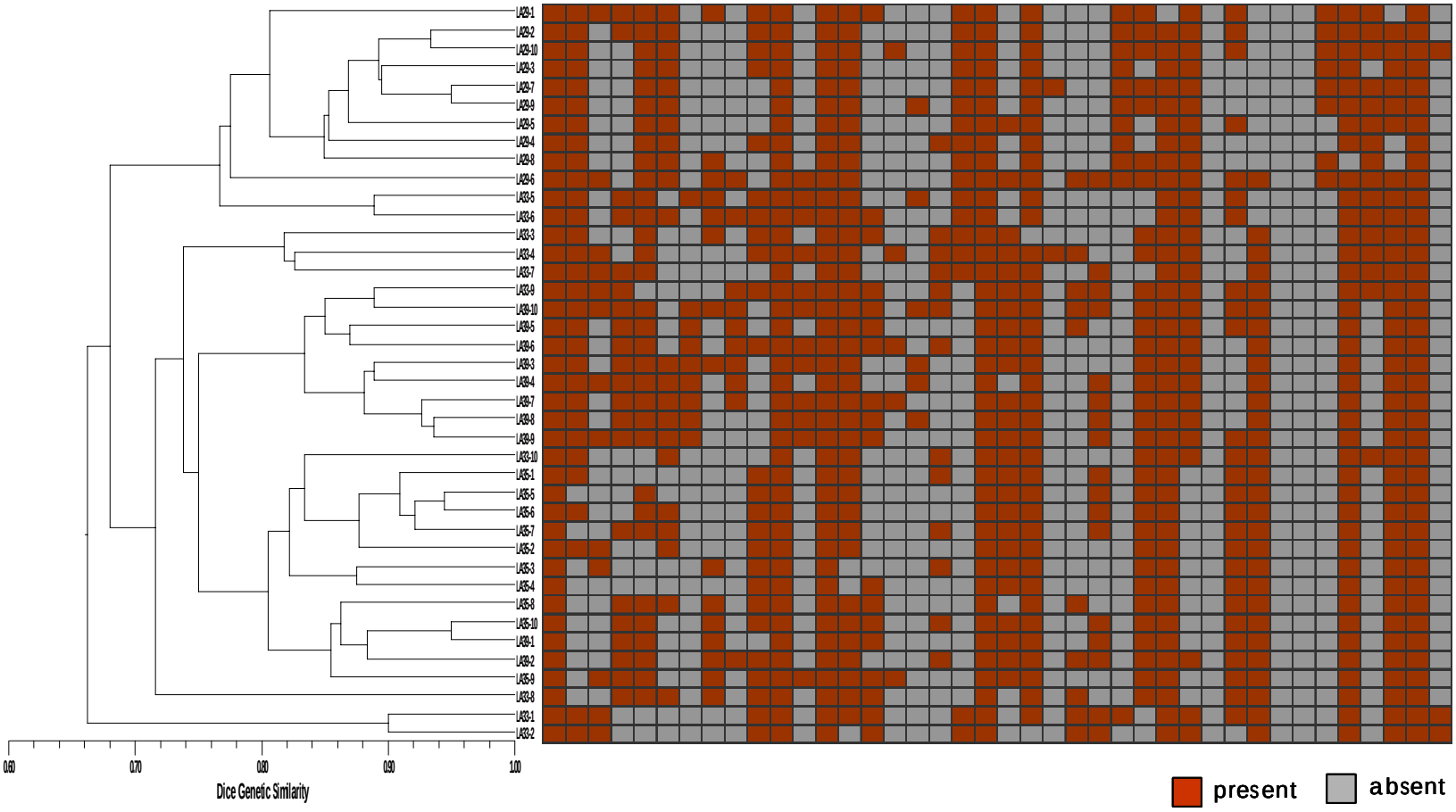


Figure 4.23 Genetic similarity and AFLP fingerprints of individual plants belonging to 4 Florida accessions (LA29, LA33, LA35 and LA39) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

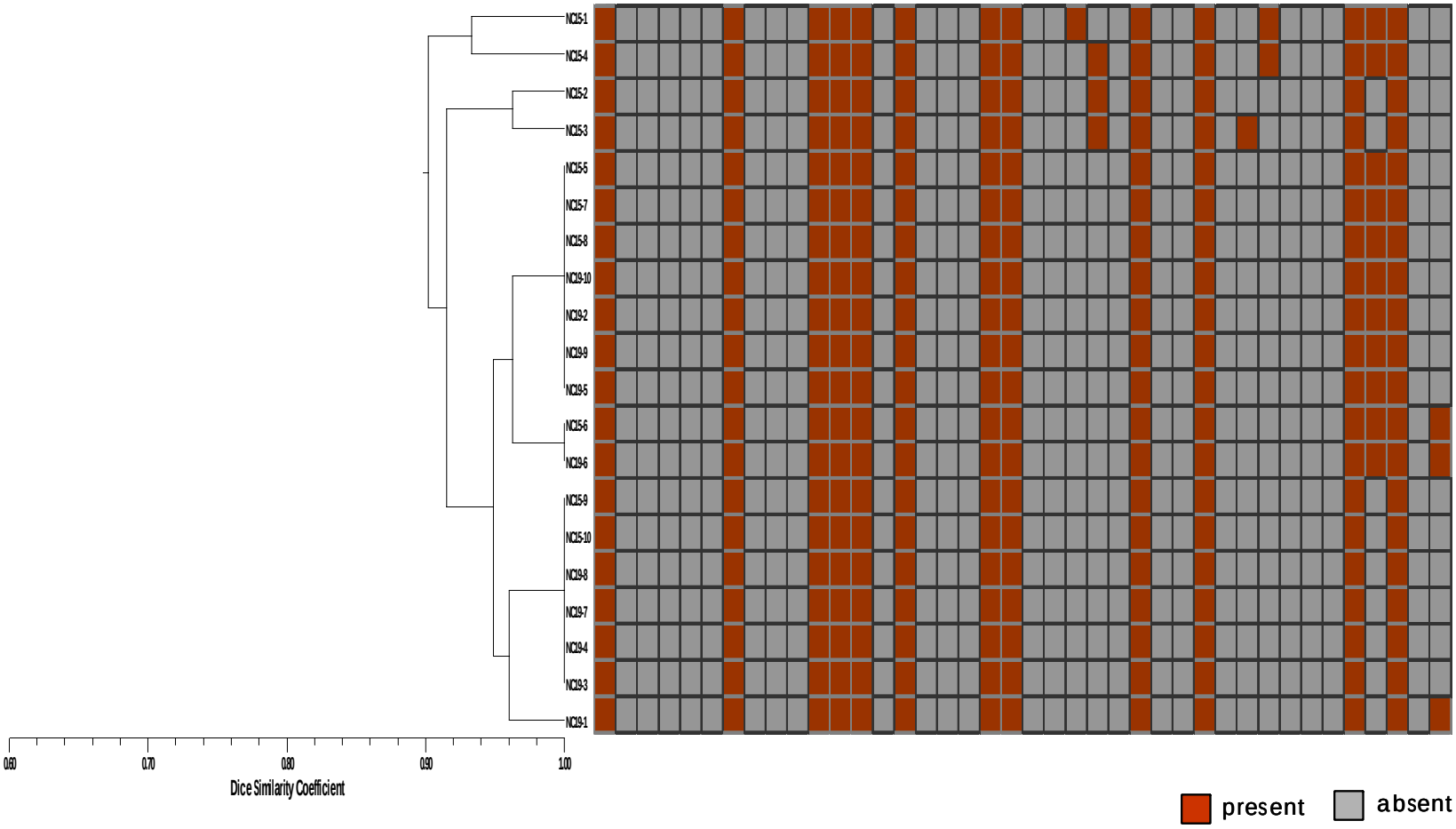


Figure 4.24 Genetic similarity and AFLP fingerprints of individual plants belonging to 2 South Carolina accessions (NC15 and NC19) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

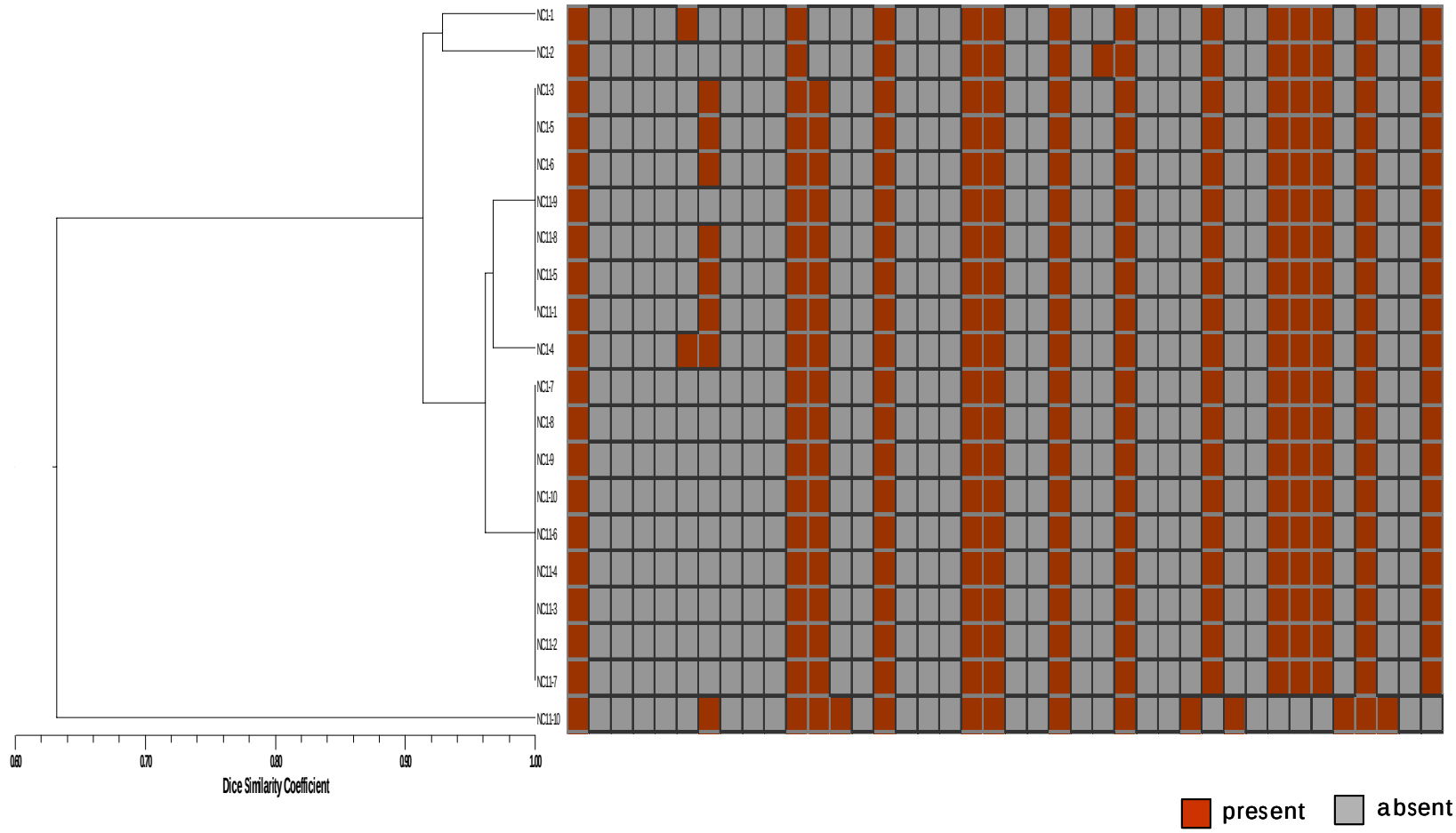


Figure 4.25 Genetic similarity and AFLP fingerprints of individual plants belonging to 2 North Carolina accessions (NC1 and NC11) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

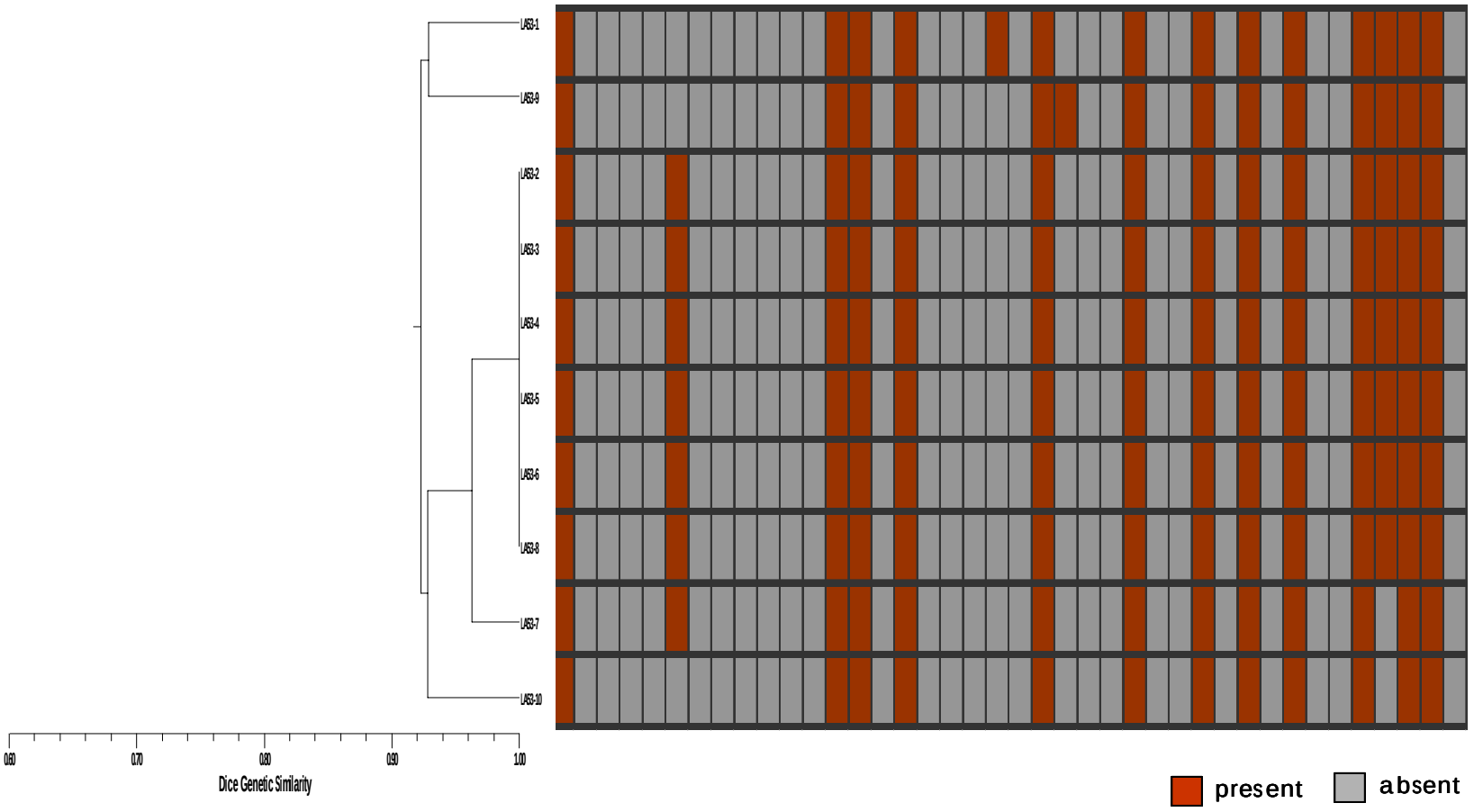


Figure 4.26 Genetic similarity and AFLP fingerprints of individual plants belonging to 1 Virginia accession (LA53) as revealed by *Eco*RI-CAG+*Mse*I-CGA primer combination.

Careful visual analysis shows that in Texas, three accessions (LA2, LA9 and LA17) each have unique AFLP bands resolved by the three primer combinations (LA2 by *Eco*RI-ACT+*Mse*I-CTC; LA9 by *Eco*RI-ACT+*Mse*I-CTC and *Eco*RI-AGG+*Mse*I-CGT; LA17 by *Eco*RI-ACT+*Mse*I-CTC). In Louisiana, LA15 has a unique AFLP band resolved by *Eco*RI-CAA+*Mse*I-CGT. Mississippi accessions (LA41 and LA47) each have unique distinct bands resolved by the two primer combinations (LA41 by *Eco*RI-AGG+*Mse*I-CAC; LA47 by *Eco*RI-AGG+*Mse*I-CGA). Florida's LA35 has two distinct AFLP band from *Eco*RI-AGG+*Mse*I-CAC while LA39 has one from *Eco*RI-AGG+*Mse*I-CAC. North Carolina's NC1 has two distinct bands resolved by *Eco*RI-AGG+*Mse*I-CAC and *Eco*RI-AGG+*Mse*I-CCT while South Carolina's NC15 has one band by *Eco*RI-AGG+*Mse*I-CCT.

4.3.11 *Uniola paniculata* L. accessions from Louisiana and Virginia

Louisiana comprises two accessions (LA15 and LA16) which both came from Fourchon Beach. These two accessions are known to be native populations of *U. paniculata* in Louisiana. Results from the AFLP fingerprinting and genetic similarity analysis showed that LA15 is a distinct accession from LA16. The individual sampled plant is consistently clustered according to its accession ID (Figure 4.12). The genetic variation observed as resolved by the 12 primer combinations in LA15 ranges from 85-92% while LA16 is from 88-93%. Furthermore, 1 AFLP marker is distinctly present in LA15 and not in LA16 as resolved by *Eco*RI-CAA+*Mse*I-CGT. Overall, the Louisiana accessions have a good range of genetic variation (85-93%).

Although the Virginia accession (LA53) is clustered in Group I together with those from Texas, Louisiana, and Florida, LA53 is a completely distinct accession from the rest. There is no single plant from Virginia that overlapped with those from other accession and

the individual plants in LA53 are grouped according to its accession assignment (Figure 4.18). The genetic similarity of LA53 is quite high compared to other accessions in Group I while its genetic variation is significant lower. The clustering of Virginia next to Texas and Louisiana can be attributed to the presence of AFLP bands common to these states. There is good indication that LA53 might have been originally collected from the southeastern areas and now is grown in Virginia. The accession-wise difference between LA53 and those other accessions from Texas, Louisiana and Florida is significantly different based on the analysis of molecular variance.

4.4 Implications for conservation and breeding

The knowledge of the genetic diversity is essential for their survival, ecology, management and development of appropriate germplasm for a diverse set of environments The information provided in this study is useful for genetic improvement of *U. paniculata* because field performance data coupled with this molecular information will delineate important differences between strains of sea oats in a given area and given population, leading to better strain selection for a specific environment. There are many traits such as adaptation, vigor, survival, and seed set which need immediate attention, to accelerate the utilization of this species in ongoing coastal restoration project in Louisiana. With the availability of information on the relatedness among the germplasm collections, appropriate germplasm can now be selected for breeding and genetic investigations.

The process by which conservation might be carried out begins with defining the populations and the areas of interest. This molecular genetic analysis would provide conservation scientists and ecological managers with new insights regarding the extent of diversity of individuals within and between populations. The genetic structure is the primary

consideration in the development of conservation strategies. In this study, we determined the relatedness and obtained information on population structure and genetic diversity of *U. paniculata* using AFLP. The AFLP analysis appeared to be efficient in verifying its diversity status. In the actual application, the measurement of genetic differences among these populations is needed to guide the on-going transplantation of *U. paniculata* to restore dune ecosystem while ensuring that a substantial genetic diversity is preserved within the study areas. For the sea oat nursery industry, this study can be useful to eliminate duplication in of the clones and ensure the diversity of the propagated clones for transplantation.

CHAPTER 5
SUMMARY AND CONCLUSIONS

With the need to prioritize plans set for natural resource management, it is empirical to understand the genetic structure of *U. paniculata* and its significance. As such, this issue of significance attribute is addressed. The results show accessions belonging to the same geographic locations significantly display differences in its genotypic profiles. Furthermore, we were able to characterize *U. paniculata* accessions that represent and embody a diverse genetic composition and structure (allelic and genotypic frequencies) within a given location.

Genetic structuring of closely related genotypes was achieved from utilizing the AFLP fingerprints generated from the twelve primer combinations. Results from this study have significantly established three major groupings (Group I, II and III) of genetically similar plants in relation to its geographic origin. UPGMA-SAHN approach distinguished one group from another based on the similarity of its AFLP fingerprints. The dendrogram shows Texas, Louisiana, Virginia and Florida accessions are grouped (Group I) as closely similar plants. Group II has the accessions from Mississippi, Alabama and the remaining two accessions from Florida while Group III mainly of the accessions from South Carolina and North Carolina. Genetic similarity across all genotypes was from 64% to 94%. The principal component analysis further confirmed the results from the cluster analysis. Meanwhile, the use of *Eco*RI-CAG+*Mse*I-CGA combination has clearly characterized the distinct fingerprints of the 190 plants differentiating the individuals from its assigned accession. The total variation within individual plants was statistically significant (34%) suggesting differences among the genotypes as manifested by the AFLP markers.

The analysis of molecular variance (AMOVA) determined the significant variations in all three levels (among states, among accessions within states and within individual

plants). The highest significant variation was noted among the states, which suggest a genetic variation influenced by isolation-by-distance. It indicates that this pattern is expected to preserve for some time and even allow a further build up of variation in the genetic composition of *U. paniculata* that colonized these widely separated areas. The difference among the accession within each state was significant (19%). Significant differences of *U. paniculata* accessions was further confirmed by pairwise comparison and permutation tests at p=0.05 as shown in *Fst* P values significance matrix.

The present study is first to establish the genetic structure of *U. paniculata* in the southeastern Atlantic and Gulf coasts of the continental United States. The results we gathered have provided the very first information on the genetic diversity of *U. paniculata* using AFLP. Our result is important for the ongoing coastal restoration project. It would help guide coastal managers to fill gaps in areas where genetic variation is low. It would primarily guide the management of germplasm collections and at the same time strengthens strategies for the most effective conservation of this plant genetic resource.

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APPENDICES

LIST OF MATERIALS, ADAPTERS AND PRIMER SEQUENCES NEEDED FOR AFLP ANALYSIS

Appendix 3.1 List of materials, adapters and primer sequences needed for AFLP analysis.

A. Equipment and materials

1. 0.2 mL PCR tubes
2. Genomic DNA
3. Thermal cycler
4. High voltage power supply (BioRad)
5. Sequencing gel unit (BioRad)

B. *Eco*RI adapter and primer sequences

1. *Eco*RI-adapter, 5 pmol/µl: 5'-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA -5’
2. *Eco*RI-primer +0, 50 ng/µl: 5’ - GACTGCGTACCAATTC -3’
3. *Eco*RI-primer +3, 50 ng/µl: 5’ - GACTGCGTACCAATTCANN -3’

C. *Mse*I adapter and primer sequences

1. *Mse*I-adapter, 50 pmol/µl: 5’ - GACGATGAGTCCTGAG
TACTCAGGACTCAT - 5’
2. *Mse*I-primer +0, 50 ng/µl: 5’ - GATGAGTCCTGAGTAA - 3’
3. *Mse*I-primer +3, 50 ng/µl: 5’ - GATGAGTCCTGAGTAACNN - 3’

Appendix 3.2 List of solution and reagents needed for making AFLP reactions.

1. 1M Tris· HAc pH 7.5
2. 1M Tris· HCl pH 8.0
3. 1M Tris· HCl pH 7.3
4. 0.1 mM $MgCl_2$
5. 1M KCl
6. 0.5 M EDTA pH 8.0
7. 10x TE: 100 mM Tris· HCl, 10 mM EDTA pH 8.0
8. Double-distilled Sterile water
9. 100 mM DTT (Dithiothreitol)
10. 10 mM ATP (Adenosine triphosphate)
11. 5x RL (restriction-ligation) buffer: 50 mM Tris· HAc, 50 mM $MgCl_2$, 250 mM KAc, 25 mM DTT, 250 ng/µl, pH 7.5
12. 10x T4-buffer: 250 mM Tris· HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 5 mM spermidine (3HCl-form)
13. 10x PCR buffer: 100 mM Tris· HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl
14. 5 mM of a mix of all 4 dNTPs (Promega)
15. Restriction enzymes: *Eco*RI (Invitrogen), *Mse*I (New England Biolabs)
16. T4 DNA ligase (Promega)
17. *Taq* DNA polymerase (Promega)
18. Molecular weight standard (10-bp ladder, Invitrogen)

Appendix 3.3 Solutions and materials needed for running a denaturing polyacrylamide gel.

1. Urea
2. 10% acetic acid solution
3. 40% acrylamide:Bis (9:1) solution in H_2O
4. N, N, N', N'-tetramethylethylenediamine (TEMED)
5. Ammomium persulphate
6. Bind silane (Sigma®)
7. Repel silane (SigmaCote®)
8. Loading buffer: 10x TBE (1 M Tris· base, 1 M boric acid, 20 mM EDTA)
9. Loading dye: 0.5 M EDTA pH 8.0, Bromophenol blue, Xylene cyanol, Deionized formamide

Appendix 4.1 Solution needed for silver staining.

Fix/stop solution	Silver stain solution	Developer solution
200 ml Acetic acid	3 ml Formaldehyde	60 g of Sodium carbonate
1800 ml of distilled water	2 g Silver nitrate	3 ml Formaldehyde
	2000 ml nanopure water	40 mg Sodium thiosulphate
		2000 ml nanopure water

VITA

Neil P. Parami worked with the European Union Development Programme-WESAMAR (1999-2001) under the Philippine Department of Agriculture as a community development officer directly involved in providing technical services to seaweed farmers in the development of the local seaweed enterprise in Samar, Philippines. He was previously connected with Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC-AQD) in Iloilo, Philippines (1994-99) as a research assistant in a seaweed project and in coastal fisheries resource management program. His first job was with the coastal environment program of the Philippine Department of Environment and Natural Resources (1993-94) as a marine biologist.

Awarded with a full scholarship grant from Fulbright, he is currently finishing his graduate thesis research in plant molecular genetics utilizing the molecular marker systems in Louisiana State University, U.S.A.

He co-authored papers published in the *Journal of Phycological Research* and *Aquaculture*. He presented in some conferences like the 1998 XVIth International Seaweed Symposium in Cebu City, Philippines and the 2003 11th Plant and Animal Genome Conference in San Diego, California. A presentation was submitted for the 12th Plant and Animal Genome Conference to be held on 2004.

Born September 25, 1971, in Ozamis City, Philippines. He is the eldest of the all-male siblings. He received his B.S. in marine biology in Mindanao State University at Naawan, Philippines on April 1993 and earned few graduate credit hours in fisheries biology at the University of the Philippines in the Visayas.