QUANTIFYING SELFING AND OUTCROSSING FERTILITY IN LOWLAND SWITCHGRASS POPULATIONS USING SSR MARKERS

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USING SSR MARKERS

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Abstract:

Switchgrass (*Panicum virgatum* L.), a native C4 perennial species, is being developed as a major cellulosic crop for biofuel feedstock production in the U.S. However, no information is available on mating behavior of plants under open pollinating conditions in the field. Accordingly, the objective of this study was to quantify selfing and outcrossing rates of switchgrass plants grown in the field. Two small (NL94 C2-3 and SL93 C2-3), each having five parents, and two large (NL94 C3 and SL93 C3), each having 26 parents, lowland switchgrass breeding populations field established with three replications were used in the experiment. Ten seedlings from open-pollinated seeds of each parent in each replication per year were planned to grow in a greenhouse at the Agronomy Research Station, Oklahoma State University. In 2010, DNA samples were isolated from 1700 progeny of 62 seed parents while DNA samples were extracted from 773 progeny of 42 parents in 2011. Sixteen Simple Sequence Repeat (SSR) markers were used to identify breeding origins of the progeny plants as compared with respective seed parents. Among 2473 progeny examined over two years, only one plant of SL 4×4 was identified to be selfed, indicating an extremely high outcrossing rate of 99.96%. The findings should help to better understand the sexual reproduction characteristics of lowland switchgrass and the identified selfed progeny could be useful in inbred line development.

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CHAPTER I

INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial grass native to North America. Switchgrass has numerous benefits as a bioenergy feedstock crop and it is widely used in soil and water conservation, as a pasture grass and for hay production (Rinehart, 2006). In recent two decades, switchgrass research has been intensified due to its potential use for bioenergy feedstock production.

Increasing oil prices and growing concerns on climate change drive investment in research on finding new energy sources. Bioenergy is an alternative source of energy that is produced from crops such as sugar cane and corn, or environmentally friendly perennial grass species, such as switchgrass (McLaughlin et al., 1999). Biofuels from biomass have the potential to reduce the consumption of fossil oil. The Bioenergy Feedstock Development Program (BFDP) at Oak Ridge National Laboratory was developed to evaluate and select the promising feedstock as sources of bioenergy for national energy needs (Martinez-Reyna and Vogel, 2008).

In the 1990s, the U.S. Department of Energy selected switchgrass as a herbaceous model for sustainable bioenergy development (Martinez-Reyna and Vogel, 2008). Switchgrass has advantages over annual crops for cellulosic biomass production because it does not have the annual establishment requirements with associated economic and net

energy input. The species is distributed across a wide geographic range demonstrating its wide adaptation. It tolerates diverse growing conditions on marginal quality land and requires low water and fertility input (McLaughlin et al., 1999).

To improve the economic value of switchgrass as a biomass energy crop, it is crucial to develop new cultivars with greater biomass yields (McLaughlin et al., 1996). To date most of the switchgrass cultivars released are improved populations or synthetic cultivars that were developed using breeding methods that utilize additive genetic variation (Taliaferro, 2002). Research has been conducted to determine whether heterosis occurs for improving biomass yield in first generation single- and double-cross progeny populations (McLaughlin et al., 1999).

Heterosis is the biological phenomenon that exhibits F1 hybrid superior performance over its parents. It has been observed in many crops and the utilization of heterosis has contributed tremendously to the increased productivity in maize and rice (Garcia et al., 2008; Zhou et al., 2012). Heterosis has been reported in switchgrass (Martinez-Reyna and Vogel, 2008; Vogel and Mitchell, 2008). Inbred lines are expected to play a fundamental role in breeding heterotic cultivars in switchgrass. Switchgrass is an allogamous species (Talbert et al., 1983). One recent experiment indicates the presence of self-incompatibility mechanisms leading to producing very little or no seed when self-pollinated (Martinez-Reyna and Vogel, 2002). However, many perennial species often are not completely outcrossing, exhibiting some selfing traits (Schemske and Lande, 1985). Taliaferro and Hopkins observed a selfing rate of less than 1% in switchgrass (Taliaferro and Hopkins, 1996; Taliaferro et al., 1999). Liu and Wu (2011)

reported that in 456 progeny from the NL94, 279 progenies (61.2%) resulted from self-fertilization in a growth chamber environment.

A mating system describes the way in which a given population reproduces sexually and it plays an important role determining how to transfer genetic information from one generation to the next (Brown and Allard, 1970). The primary mating systems in plants include outcrossing, selfing, and apomixsis (Jarne and David, 2008). Traditional methods of assessing the mating system have been based on observations on various features of floral morphology, on the behavior of pollinators, or on the results of controlled crosses (Shaw et al., 1981). However, the information derived from these methods is inadequate for quantitative estimates of mating-system parameters and is unable to provide direct measures of success of matings in populations. In the last three decades, plant geneticists began to use molecular marker tools to obtain quantitative estimates of mating system parameters.

Microsatellite, alternatively known as simple sequence repeat (SSR), has become a useful molecular tool in various aspects of molecular genetic studies in the past decade, including assessment of genetic diversity, genetic linkage mapping, QTL analysis, and marker-assisted selection in important crops, such as cotton (Liu et al., 2000), barley (Zietkiewicz et al., 1994), wheat (Zhou et al., 2008) and sorghum (Hash et al., 2003). SSR markers are repeats of short nucleotide sequences, usually equal to or less than six bases per core repeat in length, that vary in number. SSR markers are highly polymorphic, abundant, easy to use, and have become an important marker system in switchgrass genetic diversity studies (Narasimhamoorthy et al., 2008) and genetic linkage

mapping (Liu et al., 2012; Okada et al., 2010). A large number of SSR markers have been developed in switchgrass (Tobias et al., 2006; Tobias et al., 2008; Wang et al., 2011).

To estimate mating system parameters, progeny array approach (PAA) is commonly used which is based on the comparison of band pattern between maternal plant and progeny. To date no information is available on mating behavior of switchgrass plants under open pollinating conditions in the field. Accordingly, the objective of this study was to quantify selfing and outcrossing rates of lowland switchgrass plants grown in the field and to identify the selfed progeny which would be valuable for switchgrass inbred line development.

CHAPTER II

REVIEW OF LITERATURE

Significance of switchgrass for bioenergy

With the increase in oil prices and concerns about environmental issues, the United States of America has invested significant resources to develop biofuels as fuel substitutes of gasoline for sustainable development and national energy security. Ethanol is the most widely used liquid biofuel and can be produced from feedstock sources, such as sugar, starches or from cellulosic biomass. The production and use of ethanol for fuel is an effective way to decrease the dependency on fossil oil and reduce greenhouse gas emission (Demirbas, 2007). According to the advantages of bioethanol, many countries are dedicated to conducting research and innovating advanced technology towards the conversion from cellulosic biomass to ethanol. In 2005, total world ethanol production was 12.2 billion gallons, 70% of which was produced by the US and Brazil (Martines-Filho et al., 2006). In Brazil, about 4.2 billion gallons of ethanol are made annually from sugar cane (Goldemberg, 2007). The current biofuels industry in the US is based almost entirely (98%) on conversion of corn to ethanol (Petrulis et al., 1993). Numerous studies indicated that the conversion of corn into ethanol energy was negative (Pimentel and Patzek, 2005). It takes a lot of energy to convert corn into ethanol, such as irrigation,

fertilizer, pesticide, and herbicide (Patzek et al., 2005). Moreover, ethanol production using corn grain required 29% more fossil energy than the ethanol fuel produced (Pimentel and Patzek, 2005). In addition, growing large amounts of corn necessary for ethanol production needs substantial cropland suitable for food production and is questioned to cause new problems (Varvel et al., 2008).

Ethanol or other biofuels can be made from cellulosic materials such as wood, grass and wastes as well (Lynd et al., 1991). The National Bioethanol Program aims to develop technology, which can produce ethanol from the sugars in cellulose and hemicelluloses. This provides a promising future for a wide range of feedstock materials to be supplementary with current ethanol production from corn and even better to be a substitute for corn to produce the ethanol. The Biomass Feedstock Development Program at Oak Ridge National Laboratory (ORNL) funded by US-DOE initiated a series of research in 1992 to develop switchgrass as a major cellulosic biomass energy feedstock (Sanderson et al., 1996).

Biological characteristics of switchgrass

Switchgrass is a perennial species that is native to North America and it has grown in the Great Plains for centuries. The plant grows 3 to 10 feet tall with an extensive root system (Mitchell et al., 2012). Once established, well-managed switchgrass for biomass should have a productive life of 10 years or longer (Garland, 2010). It is traditionally planted for pasture and hay production, soil and water conservation and wildlife habitat (Mitchell et al., 2012).

Switchgrass is a C4 species, fixing carbon by multiple metabolic pathways with high water use efficiency (Koshi et al., 1982; Moss et al., 1969). Switchgrass is adapted to a wide geographic range, covering most of the continental U.S., east of the Rocky Mountains and extending into Mexico and Canada (Parrish et al., 2008). Natural selection combined with environmental variation due to latitude, altitude, soil type, and precipitation have resulted in significant genetic and phenotypic variation in switchgrass. Switchgrass is a highly polymorphic species, and it has a ploidy series from 2n=2x=18 to 2n=12x=108 with two major cytotypes, lowland and upland (Porter Jr, 1966). Upland types are mainly octoploids (2n=8x=72) and tetraploid (2n=4x=36), typically shorter and generally found on upland sites (Sanderson et al., 1996). Lowland switchgrass is tall, very robust and found in more moist low areas. Lowland types are predominately tetraploids (2n=4x=36) (Barnett and Carver, 1967). Lowland types have exceptional biomass yields and perform well in areas where there is a longer growing season with warmer temperatures such as the Southern USA (Bouton, 2007; Porter Jr, 1966). Within these two major cytotypes, further subdividing into southern lowlands and northern lowlands; southern uplands and northern uplands is also reported according to latitudinal adaptation (Casler et al., 2004; Casler et al., 2007).

Breeding switchgrass for bioenergy feedstock production

Switchgrass is an outcrossing species and sexually reproduced by seed. Cultivars are expected to be either: broad genetic base plant populations; synthetics compromising 2 to 12 selected parent plants; or F1 hybrids (Taliaferro et al., 2000). To improve economic value of switchgrass as a biomass energy crop, many efforts were made to maximize potential biomass yields (Sanderson et al., 1996). Initial switchgrass cultivar

development focused on accession or ecotype collections, screening the performance and geographic adaptation in field trials and then releasing the best accession population as a new cultivar (Vogel, 2004).

More research activities were performed to develop experimental synthetic cultivars using elite parent plants from breeding populations (Taliaferro, 2002). It is technically feasible to produce F1 hybrids, however, no commercial F1 hybrid switchgrass cultivar has been produced (Taliaferro et al., 2000). Martinez-Reyna and Vogel (2008) reported the hybridization in switchgrass spaced plants between 'Kanlow', a lowland tetraploid and 'Summer', a upland tetraploid. Research on tissue culture techniques for clonal reproduction of parent plants also accelerates the breeding process for desired or selected genotypes. A previous study reported a micropropagation procedure was developed with nodal segments and produced hundreds of plantlets from a single parent plant of switchgrass in a period of three months (Alexandrova et al., 1996). This technique now makes possible rapid development of isolated breeding blocks of superior plants for developing narrow genetic base synthetics as well as F1 hybrids (McLaughlin et al., 1999).

Heterosis and inbred lines in switchgrass

Heterosis or hybrid vigor refers to the performance of progeny that exhibit greater biomass, speed of development and fertility compared to both parents (Birchler et al., 2010). Heterosis has been successfully employed by corn cultivar development resulting in high yield and uniform hybrids (Bouton, 2007). Hybrid maize development improves farmers' productivity and helps ensure a reliable, sustainable food supply (Bouton, 2007).

Corn hybrid plants are produced by crossing homozygous inbred lines. Homozygosity in an inbred line is achieved by repeated inbreeding and in general the inbred line is considered genetically pure by the sixth or seventh generation of continuous selfing (Troyer, 1986). The selection and production of inbred lines are extremely important to provide superior F1 hybrids. Hybridizing inbreds may have the potential to dramatically increase the biomass yield of switchgrass. Heterosis has been reported in switchgrass (Martinez-Reyna and Vogel, 2008; Vogel and Mitchell, 2008). As switchgrass is a naturally outcrossing species, the identification of selfed progeny is important to producing inbreds that can be used in the production of heterotic hybrids. However, only first generation of selfed progeny was reported in one population (Liu and Wu, 2011).

Self-incompatibility and mating behavior of switchgrass

Switchgrass, an open-pollinated species, produced very little or no seed when self-pollinated indicating the presence of incompatibility mechanisms. Self-incompatibility in some grasses is determined by the action of two independently segregating polyallelic loci, S and Z. The pollen grain is specified gametophytically by the complementary interaction of S and Z genes. A pollen grain will be incompatible with a style that has the same alleles (Martinez-Reyna and Vogel, 2002). Mating system is one of the major attributes of the reproductive biology of a plant species and it determines how genetic information is transferred from one generation to the next generation (Schoen and Clegg, 1984). A mating system is a way in which a group is structured in relation to sexual behavior, which describes the proportion of matings between related individuals or the proportion between unrelated individuals within a population (Barrett, 2002). Selfing occurs when both the pollen and ovule are produced by the same

individual (Dudash and Murren, 2008).The primary mating systems in plants are outcrossing, selfing, and apomixsis.

The mixed-mating model is in a central position in both theoretical and experimental investigations in plant population genetics. Mixed-mating model was chosen to describe the pattern of gene transmission in plant populations due to several reasons (Ritland, 2002). First, inbreeding exists in many plant species and therefore the random-mating model is inappropriate. Second, mixed-mating model is simple and only requires the estimation of selfing rate and outcrossing rate. Third, mixed-mating model is a reasonable description of the reproductive biology in many species (Schoen and Clegg, 1984). Mixed mating is appropriate in hermaphrodite plant species that reproduce by both self- and cross-fertilization and quantitatively describes the basic mating system parameters, such as outcrossing, selfing and inbreeding coefficient (Goodwillie et al., 2005).

Reliable information on mating system is essential to understand how genotypic frequency is transformed over generations and in an evolutionary environment. Traditional methods of assessing the mating system have been based on observations on various features of floral morphology, on the behavior of pollinators, or on the results of controlled crosses experiments (Shaw et al., 1981). However, the information derived from these methods is inadequate for quantitative estimates of mating-system parameters and is influenced by environmental factors and is unable to provide direct measures of mating type in populations.

Conventional plant breeding is time consuming and dependent on environmental conditions. Therefore, breeders are extremely interested in new technology that makes this procedure more efficient (Korzun, 2002). Molecular markers have been widely developed as a genetic tool for plant genotyping and gene mapping; it is also applied to investigate the genetic variation of plants in the past few decades. Molecular markers provide opportunities to improve the research progress by using a series of novel approaches.

Molecular markers used in experiments to quantify mating systems

In recent years, different markers systems have been developed and applied to investigate the mating systems in a range of plant species. The three most commonly used markers in evaluation of mating systems are allozymes, amplified fragment length polymorphism (AFLP) and microsatellites (Jarne and David, 2008). Traditionally isozymes have been utilized for this purpose (Epperson and Allard, 1984; Politov and Krutovskii, 1994). Kittelson and Maron (2000) estimated an outcrossing rate of 0.78 in *Lupinus Arboreus* based on four isozyme loci from 34 maternal progeny arrays of seeds. Allozymes at two loci were used for progeny arrays grown from the open-pollinated seed to calculate outcrossing rate in alfalfa in easy-to-trip population (0.76), hard-to-trip population (0.75) and the CUF101 parent population (0.77) (Knapp and Teuber, 1993).

AFLPs are dominant markers with two alleles per locus (present/absent) if the organism is a diploid, although the probability that different bands actually represent alleles of the same locus is generally unknown (Mueller and Wolfenbarger, 1999). These limitations might, to a certain extent, be counterbalanced by the large number of loci

scored. Muluvi et al. (2004) estimated the outcrossing rate of 0.74 in a mixed mating system study of *Moringa oleifera* using AFLP markers. Muluvi et al. (2010) reported the outcrossing rate of 89% in *Warburgia ugandensis* using the mixed mating model with AFLP markers.

Microsatellites, alternatively known as simple sequence repeat (SSR) markers have become a useful molecular tool in various molecular genetic studies in the past decade, including assessment of genetic diversity, genetic linkage mapping, fingerprinting, and marker-assisted selection (Rakoczy-Trojanowska and Bolibok, 2004). SSR markers are repeats of short nucleotide sequences, usually equal to or less than six bases in length, that vary in number. SSR markers are codominant, are highly polymorphic, abundant, and easy to use (Hayden and Sharp, 2001). Using six SSRs, Muraya et al. (2011) estimated the outcrossing rates of 12 wild sorghum populations in four sorghum growing regions and indicated that wild sorghum in Kenya exhibits a mixed mating system and the crossing rate is affected by ecological factors. de Souza et al. (2012) estimated the cross-pollination rate using SSRs to support the hypothesis of frequent allogamy with high rates of outcrossing in two olive genotypes.

Previous studies indicated the rate of self-pollination in switchgrass varied greatly from less than 1% (Martinez-Reyna and Vogel, 2002; Taliaferro and Hopkins, 1996) when its inflorescences were bagged to higher than 60% when two plants were grown in a growth chamber (Liu and Wu, 2011). Identification of selfed progenies is very useful to develop advanced inbred lines which can serve as parents for F1 hybridization. Liu and Wu (2011) confirmed selfed progeny in switchgrass with 12 simple sequence repeat (SSR) markers. It is time consuming if a large amount of progeny are genotyped. A

duplex PCR protocol of SSR markers sampling much of the switchgrass genome has been reported recently (Liu and Wu, 2012). Evidently, a duplex will save approximately 50% of time as compared with two separate PCR reactions.

Switchgrass is a perennial tall grass and selected by the U.S. Department of Energy as a model herbaceous energy crop. Breeding protocols can affect improvement of forage yields of switchgrass. The development of hybrid switchgrass cultivars is possible with the laboratory culture techniques and the strong self-incompatibility of switchgrass. The homozygous switchgrass inbred lines are extremely valuable to serve as parents to produce F1 hybrids. However, since the presence of self-incompatibility in switchgrass, it is very challenging to produce the selfed progenies. Selfing rate is influenced greatly by ecological and genetic factors. A previous study reported that a NL 94 population genotype grown in a growth chamber had a self-fertilization rate of more than 60% (Liu and Wu, 2011). However, no information is available on mating behavior of switchgrass plants under open pollinating conditions in the field.

CHAPTER III

METHODOLOGY

Experimental site, materials and experimental design

Switchgrass plantings used in this study were field established at Cimarron Valley Research Station at Perkins, Oklahoma. According to Oklahoma Ag Experiment Station Field and Research Service Unit website (http://www.oaes.okstate.edu/field-and-research-service-unit/agronomy-research-station-perkins-1/Cimarron%20soil%20map.pdf/view), the soil type was teller fine sandy loam.

Four lowland switchgrass populations were used in this study: two genetically narrow-based populations, SL93 C2-3 (SL93 and SL used interchangeably) and NL94 C2-3 (NL94 and NL used interchangeably), each population having five parental genotypes with 3 replications respectively; and two genetically broad-based populations, SL93 C3 and NL94 C3, each comprising 26 parental plants with three replications. All southern lowland plants were selections from SL 93 C-1 selection nursery and all northern lowland plants were from NL 94 C-1 selection nursery. Both SL93 C-1 and NL94 C-1 nurseries were space-planted in separate field plots at Lake Carl Blackwell in 2001 and selections were made in 2008. Switchgrass parental clones of SL93 C2, NL94 C2, SL93 C2-3 and NL94 C2-3 populations were prepared in a greenhouse in the winter between 2008 and 2009, and transplanted on 1m centers into

four experimental plots in the spring of the same year. SL93 C2-3 and NL94 C2-3 were planted on April 09, 2009 while SL93 C2 and NL94 C2 were established on April 22 in the same year. Therefore, the experimental design was a randomized complete block design (RCBD) with three replications.

Field management

After transplanting clonal plants into field plots at Perkins, water was supplemented to ensure newly grown plants survive. The four plots were fertilized with 67.5 kg nitrogen/hectare in May of 2009, 2010 and 2011. Weeds were controlled by an application of 1.1 kg active gradient of atrazine (pre-emergence herbicide) per ha in spring annually. Plant residues in the plots were burned before greening up in February of 2010 and 2011.

Seed harvesting, prechill and planting

Mature inflorescence samples were hand-harvested from each plant for every replication in each of the four plots at Perkins on Oct 1, 2010 and November 1, 2011, respectively. Each sample consisting of five morphologically mature inflorescences was kept in a separate bag in 2010. Leaf tissues for each of the 62 maternal plants were hand collected on April 27, 2011. In 2011, since the summer weather was harshly hot and dry, consequently seed set in switchgrass was low, 20 mature inflorescences were collected in an attempt to get more seeds for the research. After harvest, seeds were dehydrated for about 4 weeks at the room temperature. Then seeds were removed from the panicles by rubbing and cleaned utilizing a South Dakota Seed Blower (Seedburo Equipment Co., IL, USA). Normally, newly harvested switchgrass seeds have a high percentage of

dormancy. It is desirable to break the dormancy to achieve a higher germination rate (Teel and Barnhart, 2003). According to Haynes et al. (1997), seeds were placed on wet white filter papers soaked with a 0.2% KNO_3 in pertri dishes and stratified at 4°C in a refrigerator for two weeks, which is called prechilled procedure. After the prechill treatment, the seeds were respectively sown into black cells containing a soil mix in a greenhouse at Agronomy Research Station, Oklahoma State University. Seedlings were counted after 7 days, and each of 10 random seedlings from each sample was transplanted into one container for further growth in the greenhouse for leaf tissue collection.

DNA extraction, polymerase chain reaction and gel electrophoresis

Total genomic DNA was extracted from 0.15g fresh frozen leaf tissues of each of the progenies and their parents using the CTAB method (Wang et al., 2011). DNA concentrations were quantified using a NanoDrop DN-1000 Spectrophotometer (NanoDrop products, DE, USA). Each DNA working solution was adjusted to a concentration of 10ng/µl as the template for PCR. For PCR amplification of the samples collected in 2010, the following eight primer pairs (PVGA-1549/1550, PVCAG-2389/2390, PVCA-615/616, PVCA-815/816, SWW-1622, 5211_B07, PVAAG-3163/3164, PVGA-1143/1144) (Table 3.1) were used to genotype the maternal plants and 10 open-pollinated progeny of each maternal plants. In total, 1762 DNA samples including 62 parental plants and their open-pollinated progenies with 3 replications were genotyped with the eight SSR primer pairs in 2010. With the successful development of duplex PCR protocol in our lab (Liu and Wu, 2012), the following eight primer pairs (PVCAG-2397/2398, PVCAG-2517/2518, PVCAG-2269/2270, PVCAG-2361/2362,

SWW-2622, 5211_B07, PVAAG-3163/3164, PVGA-1143/1144) (Table 3.2) were performed on all samples collected in 2011 using the duplex-PCR protocol of Liu and Wu (2012). Putative selfed progenies were further genotyped by four additional SSR duplexes (PVCAG-2147/2148, SWW-1394, NFSG-112, NFSG-036, PVAAG-3311/3312, PVGA-1813/1814, PVCA-893/894, SWW-1615) for accurate identification (Table 3.3).

In the analysis of the samples collected in 2010, the SSR-PCR amplifications were performed according to Wu and Huang (2008). Each reaction of 10.5 µl volume consisted of 4.22µl of H_2O, 1.0 µl 10×buffer, 0.6µl 25mM of $MgCl_2$, 0.2µl 10mM of dNTP, 0.1µl 50U/µl Taq DNA polymerase, 1.34µl 1 pmd/µl forward primer and reverse primer each, 0.2µl 1µM IR-M13 primer, and 1.5 µl 10ng/ µl of template DNA. In the analysis of the samples collected in 2011, duplex-PCR amplifications were performed in a 11 µl final volume consisting of 1.64 µl of H_2O, 1µl 10×buffer, 0.96 µl 25mM of $MgCl_2$, 0.2 µl 10mM of dNTP, 0.1 µl 50U/ µl Taq DNA polymerase, 1.3 µl Forward primer and Reverse primer for each primer pair, 0.2 µl 1 µM IR-M13 primer, and 1.7 µl 10ng/µl of template DNA (Liu and Wu, 2012).

PCR reactions were performed in a 96-well PCR plate using a 2720 Thermal Cycler (Applied Biosystems, IL, USA) with the following program with an initial denaturation of 5 min at 95°C for 1 cycle, 14 cycles of 20s at 94°C, 1 min at 58°C, 30s at 72°C; 28 cycles of 20s at 94 °C, 1min at 55°C, 30s at 72°C; and a final 10 min extension at 72°C. Then the temperature decreased to 4°C. 5.0 µl blue stop solution was added to each PCR reaction well, spun down, and denatured for 3 min at 94°C in the 2720 thermal cycler (Applied Biosystems, IL, USA). The PCR products from a plate labeled with

700nm florescence dye and the other plate labeled with 800nm florescence dye (LI-COR Inc., NE, USA) were mixed together.

For scoring each genotype, the mixed amplified products were separated on 6.5%KB^{plus} LI-COR gels (LI-COR Inc., NE, USA) with a 64-tooth comb and run at 1500 volts for 1 hour and 45 min in a LI-COR 4300 DNA Analyzer (LI-COR Inc., NE, USA). A DNA marker of 50-350bp size standards (LI-COR Biosciences, Lincoln, NE, USA) was also loaded to determine the size of the amplified fragments. Bands were visually scored.

Data analysis

Progeny array approach is used to identify the selfed progeny based on the comparison between maternal parent genotypes and their respective open-pollinated progenies. The data were processed in Microsoft Excel and displayed the trend of outcrossing rate with eight SSRs from four switchgrass populations in 2010 and 2011 using bar chart.

Table 3.1 Parameters of eight microsatellite markers used in 2010

SSR marker ID	Type	Repeat motif	LG	Position	Lmin-max(bp)	Primer pmol
PVGA-1549/1550	gSSR	$(GAA)_6$	1b	83.4	270-280	1
PVCAG-2389/2390	gSSR	$(GAAGG)_4$-$(AGCAGG)_4$	7b	3.8	240-255	1
PVCA-615/616	gSSR	$(AC)_{11}$	4b	71.9	215-230	1
PVCA-815/816	gSSR	$(AC)_{27}$	2a	70	305-330	1
SWW-1622	eSSR	(GCG)n	2b	56.3	240-250	1
5211_B07	eSSR	$(AGC)_8$	2a	17.2	240-255	1
PVAAG-3163/3164	gSSR	$(ACA)_{29}$	5b	63.5	211-293	1
PVGA-1143/1144	gSSR	$(GA)_7$-$(GA)_8$	5a	29.3	156-195	1

Table 3.2 Parameters of eight microsatellite markers used in 2011

SSR marker ID	Type	Repeat motif	LG	Position	Lmin-max(bp)	Primer pmol
PVCAG-2397/2398	gSSR	$(CAG)_{12}$	3b	36.3	161-189	2
PVCAG-2517/2518	gSSR	$(GCT)_{8}$	9a	6.9	213-234	2
PVCAG-2269/2270	gSSR	$(CAG)_{8}$	4b	0	209-262	0.5
PVCAG-2361/2362	gSSR	$(AGC)_{8}$	1b	25.9	268-277	1
SWW-2662	eSSR	$(AGG)_{n}$	2b	73.5	178-197	1
5211_B07	eSSR	$(AGC)_{8}$	2a	17.2	240-253	1
PVAAG-3163/3164	gSSR	$(ACA)_{29}$	5b	63.5	211-293	1
PVGA-1143/1144	gSSR	$(GA)_{7}$-$(GA)_{8}$	5a	29.3	156-195	1

Table 3.3 Parameters of additional eight microsatellite markers used for genotyping two putative selfed progeny

SSR marker ID	Type	Repeat motif	LG	Position	Lmin-max(bp)	Primer pmol
PVCAG-2147/2148	gSSR	$(CAG)_7$	6b	150.5	285-306	1
SWW-1394	eSSR	$(GGT)_n$	7a	60.4	194-217	0.5
NFSG-112	gSSR	$(GA)_n$	8b	48	189-195	0.5
NFSG-036	gSSR	$(GA)_n$	4a	0	120-167	0.5
PVAAG-3311/3312	gSSR	$(CTT)_{28}$	2a	29.6	140-170	0.5
PVGA-1813/1814	gSSR	$(GA)_7$	5a	72.2	236-276	1
PVCA-893/894	gSSR	$(AC)_{19}$	3b	65.3	297-336	1
SWW-1615	eSSR	$(GGC)_n$	1a	109.8	185-216	1

CHAPTER IV

FINDINGS

Seed germination in 2010 and 2011

The seeds collected in 2010 germinated well, resulting in 1700 half-sib progeny with 62 female parents for the genotyping research. However, panicles harvested from the same parental plants in 2011 had poor seed set and harvested seeds had low germination. Consequently, 773 half-sib progeny of 42 female parents were developed (Tables 4.1- 4.4).

SSR markers for PCR amplification in four populations in 2010 and 2011

In 2010, eight SSR markers (Table 3.1) that were distributed in different linkage groups or the same linkage group (distance>50cM) produced clearly scorable bands with approximate sizes as published previously (Figure 4.1). The allele band size range of the eight SSR markers was from 156bp (PVGA-1143/1144) to 330bp (PVCA-815/816). In 2011, with the development of duplex-PCR in switchgrass (Liu and Wu, 2012), four duplexes of eight primer pairs (Table 3.2) that positioned on different linkage groups or the same linkage group (distance 50>cM) were utilized to amplify the clearly readable bands (Figure 4.2). The allele band size range of the 8 SSR markers was from 156bp (PVGA-1143/1144) to 277bp (PVCAG-2361/2362).

Determination of outcrossed and selfed progeny using SSR markers

All SSR markers worked effectively for amplifying target bands in the parents and open-pollinated progeny. Band patterns were scored for each parent and their progeny. Most of the progenies were judged true progeny if at least one maternal band was present. There were 60 progenies judged contaminants if target band was not from seed parents, assuming no mutation occurred.

In genotyping the progeny population of NL94 C2-3 in 2010, the identified outcrossing rate was 43.2% with one SSR, increased to 59.5% with two SSRs, 62.2% three SSRs, 79.1% four SSRs, 91.9% five SSRs, 96.7% six SSRs, 98% seven SSRs, and 100% with eight SSRs (Figure 4.3). The identified outcrossing rate of NL94 C2-3 in 2011 was 66.7% with one SSR and reached to 100% when genotyped with six SSRs (Figure 4.4). In the 2010 progeny population of SL93 C2-3, the identified outcrossing rate was 71.4% with first SSR and 100% with three SSRs (Figure 4.5). The identified outcrossing rate of SL93 C2-3 in 2011 was 99.2% with five SSRs and remained the same with additional three SSRs, indicating one progeny of parent (SL 4×4) was derived from selfing (Figure 4.6). Similarly, one progeny of parent (SL 13×6) was identified to be selfed in the progeny of SL93 C3 produced in 2010 subsequently the identified outcrossing rate was 99.9% with eight SSRs (Figure 4.7); in 2011, the identified outcrossing rate of the population accumulated to 100% (Figure 4.8). In population NL94 C3, the identified outcrossing rate was 100% in both 2010 and 2011, respectively (Figure 4.9 and Figure 4.10). To be accurate, and make sure the selfed progenies were truly from selfing, the two putative selfed progenies with their respective parents, SL 13×6 and SL 4×4, were re-genotyped with eight SSRs (Figure 4.11). Later, these two selfed progenies

were further genotyped by four additional SSR duplexes (i.e. eight SSRs) (Table 3.3) for accurate identification and one progeny of SL 4×4 demonstrated truly selfed band patterns (Figure 4.12 and Figure 4.13).

Talbert et al. (1983) reported an average selfed rate of less than 1% when seed yields of bagged inflorescences were compared with those of unbagged ones of lowland switchgrass plants. Similar results were reported by Taliaferro and Hopkins (1996). Martinez-Reyna and Vogel (2002) reported a 0.35% selfing rate in tetraploid plants and 1.39 % for octoploid plants using a similar bagging method. In this experiment, recently available SSR markers were used in the identification of selfing vs. crossing progeny in four lowland switchgrass populations under open pollination environments for two years. Our result indicated lowland switchgrass plants produced only one selfed progeny out of more than 2,400 open-pollinated progeny harvested on 62 different parents. The results indicate lowland switchgrass plants produced less selfed progeny when grown in the field than bagged inflorescences or when grown in a growth chamber. The results further indicated that lowland switchgrass is a complete or near complete outcrossing species in open-pollination environments. Martinez-Reyna and Vogel (2002) reported prefertilization incompatibility under gametophytic control as responsible for the very low selfing rate. More recently, Liu and Wu (Liu and Wu, 2011; Liu and Wu, 2012) reported much higher selfing rates when two plants were grown in a growth chamber. The higher self-fertilization rate likely resulted from the absence of pollen produced from other switchgrass plants of the same ploidy level (Liu and Wu, 2011).

Table 4.1 Seedling list of NL94 C2-3 in 2010 and 2011

LU ID	Nursery ID	Rep	Progeny	
			2010	2011
1		1	10	10
2	NLL3×7	2	10	10
3		3	10	10
4		1	10	0
5	NLL4×18	2	10	0
6		3	10	0
7		1	10	10
8	NLL7×1	2	10	0
9		3	10	0
10		1	10	7
11	NLH41×4	2	10	0
12		3	10	0
13		1	10	0
14	NLH66×12	2	10	0
15		3	10	10
		Total:	150	57

Table 4.2 Seedling list of SL93 C2-3 in 2010 and 2011

LU ID	Nursery ID	Rep	Progeny	
			2010	2011
16		1	10	10
17	SL4×4	2	10	10
18		3	10	10
19		1	10	10
20	SL13×6	2	10	10
21		3	10	5
22		1	10	10
23	SL18×23	2	10	0
24		3	10	10
25		1	10	10
26	SL27×14	2	10	10
27		3	10	0
28		1	10	10
29	SL28×16	2	10	10
30		3	10	10
		Total:	150	125

Table 4.3 Seedling list of SL93 C3 in 2010 and 2011

LU ID	Nursery ID	Rep	Progeny	
			2010	2011
31		1	10	10
32	SL1×10	2	10	10
33		3	0	0
34		1	0	0
35	SL4×4	2	0	10
36		3	0	10
37		1	10	10
38	SL4×13	2	10	10
39		3	10	10
40		1	10	10
41	SL6×28	2	10	10
42		3	10	10
43		1	10	10
44	SL7×11	2	10	0
45		3	10	10
46		1	10	10
47	SL8×25	2	10	10
48		3	10	10
49		1	10	10
50	SL10×27	2	10	10
51		3	10	10
52		1	10	10
53	SL10×30	2	10	0
54		3	10	10
55		1	10	0
56	SL12×1	2	10	0
57		3	10	0
58		1	10	0
59	SL12×20	2	10	10
60		3	10	10
61		1	10	10
62	SL13×6	2	10	10
63		3	10	10
64		1	10	0
65	SL18×23	2	10	10
66		3	10	10
67		1	10	10
68	SL19×2	2	10	10
69		3	10	10
70		1	0	0
71	SL20×18	2	10	0
72		3	0	0
73		1	10	10
74	SL21×12	2	10	10
75		3	10	10
76		1	10	0
77	SL23×28	2	10	10
78		3	10	0
79		1	10	5
80	SL25×13	2	10	10
81		3	10	10
82		1	10	10
83	SL27×14	2	10	0
84		3	10	10
85		1	10	0
86	SL28×16	2	10	0
87		3	10	10
88		1	10	0
89	SL29×5	2	0	0
90		3	10	10
91		1	10	10
92	SL30×3	2	10	0
93		3	10	0
94		1	10	10
95	SL31×5	2	10	10
96		3	10	0
97		1	10	10
98	SL31×16	2	10	10
99		3	10	10
100		1	10	0
101	SL31×22	2	10	10
102		3	10	0
103		1	10	0
104	SL32×25	2	10	10
105		3	10	10
106		1	10	0
107	SL34×23	2	10	0
108		3	10	10
		Total:	710	505

Table 4.4 Seedling list of NL94 C3 in 2010 and 2011

LU ID	Nursery ID	Rep	Progeny	
			2010	2011
109		1	10	10
110	NLH6×8	2	10	0
111		3	10	5
112		1	10	0
113	NLH13×11	2	10	0
114		3	10	0
115		1	10	0
116	NLH27×2	2	10	0
117		3	10	0
118		1	10	10
119	NLH30×1	2	10	0
120		3	10	10
121		1	0	0
122	NLH41×4	2	10	0
123		3	10	0
124		1	10	10
125	NLH51×4	2	10	0
126		3	10	1
127		1	10	7
128	NLH59×6	2	0	0
129		3	10	0
130		1	0	0
131	NLH66×12	2	0	1
132		3	10	0
133		1	10	0
134	NLL1×8	2	10	0
135		3	10	0
136		1	10	6
137	NLL1×14	2	10	2
138		3	0	0
139		1	10	0
140	NLL2×2	2	10	0
141		3	10	0
142		1	10	0
143	NLL3×7	2	10	0
144		3	10	0
145		1	10	0
146	NLL4×18	2	10	0
147		3	10	0
148		1	10	10
149	NLL7×1	2	10	0
150		3	10	0
151		1	10	0
152	NLL7×10	2	10	0
153		3	10	0
154		1	10	0
155	NLL11×14	2	10	0
156		3	10	0
157		1	10	0
158	NLL17×9	2	10	0
159		3	10	0
160		1	0	0
161	NLL18×17	2	10	0
162		3	10	0
163		1	10	0
164	NLL24×17	2	10	0
165		3	10	0
166		1	10	0
167	NLL25×12	2	10	0
168		3	10	0
169		1	10	0
170	NLL26×8	2	10	0
171		3	10	0
172		1	10	0
173	NLL26×24	2	0	0
174		3	0	0
175		1	10	0
176	NLL27×27	2	0	0
177		3	10	0
178		1	10	0
179	NLL30×27	2	10	4
180		3	10	0
181		1	10	0
182	NLL33×9	2	10	0
183		3	10	0
184		1	10	0
185	NLL34×25	2	10	0
186		3	10	10
		Total:	690	86

Figure 4.1 A gel image of SSR primer pair PVGA-1549/1550 genotyping SL93 C3 five parents and respective progeny derived from seed samples harvested in 2010. M stands for standard molecular size markers with specific sizes given on the right side of the gel. Parent samples are replicated two times (i.e. two gel lanes) and labeled with Nursery ID (see Table 4.3) in red color while individual progeny follow their parent samples.

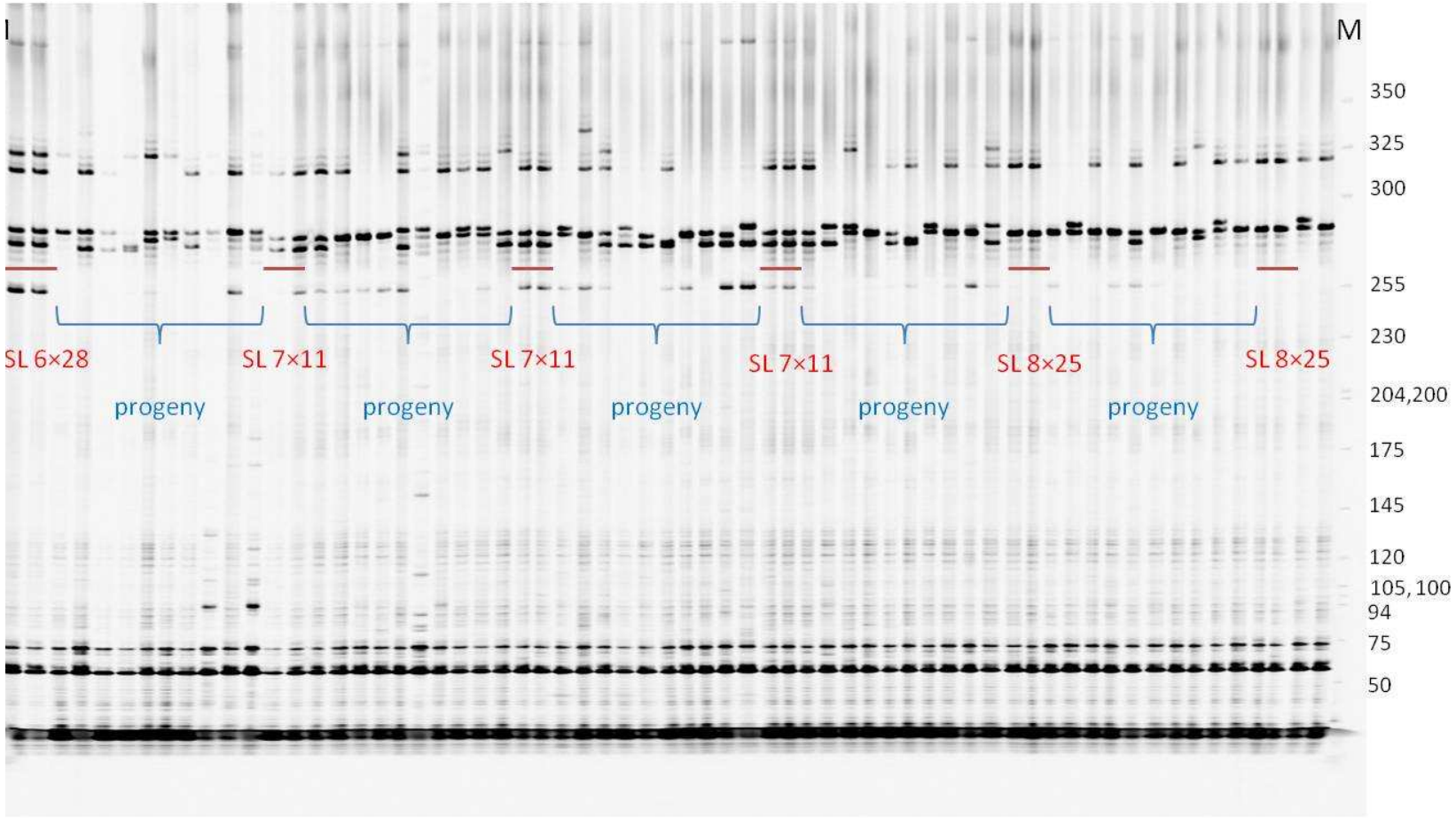


Figure 4.2 A gel image of SSR primer pairs PVCAG-2361/2362 and PVCAG-2269/2270 on SL93 C3 five parents and respective progeny derived from seed samples harvested in 2011. M stands for standard molecular size markers with specific sizes are given on the right side of the gel. Parent samples are replicated two times (i.e. two gel lanes) and labeled with Nursery ID (see Table 4.3) in red color while individual progeny follow their parent samples. The upper bands are amplified by PVCAG-2361/2362 and lower bands are genotyped by PVCAG-2269/2270.

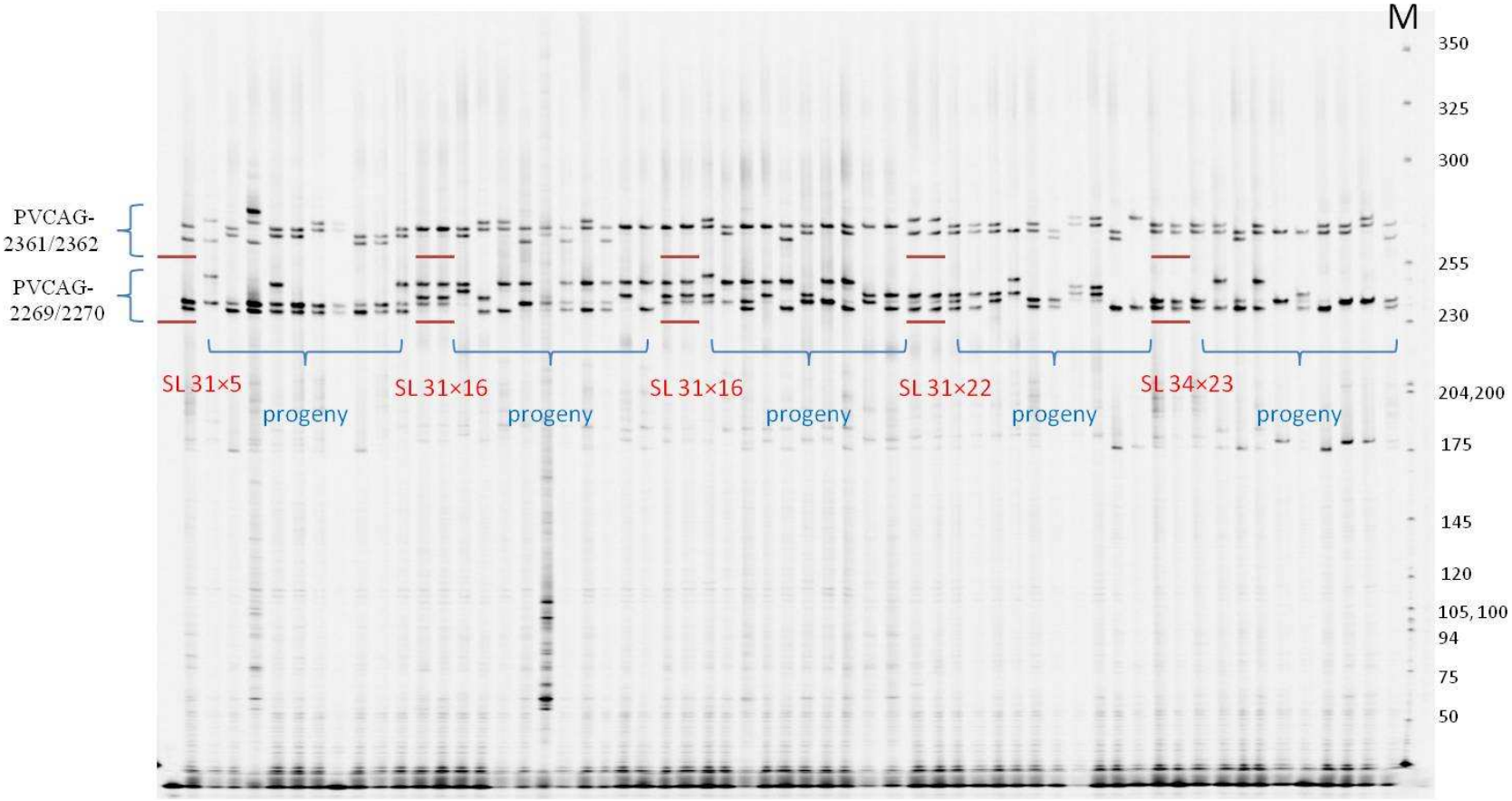


Figure 4.3 Outcrossing rates of 2010 NL94C2-3 progeny identified with eight SSR markers

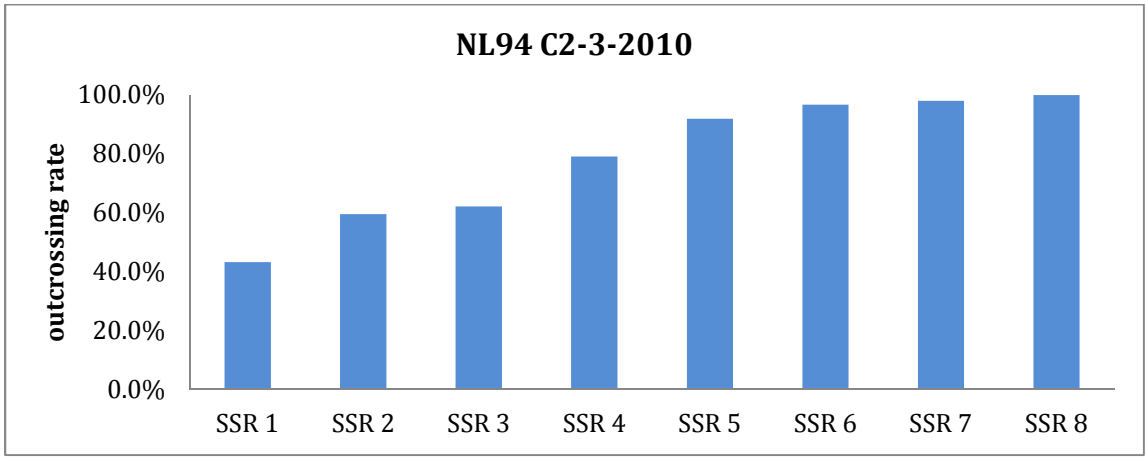


Figure 4.4 Outcrossing rates of 2011 NL94C2-3 progeny identified with eight SSR markers

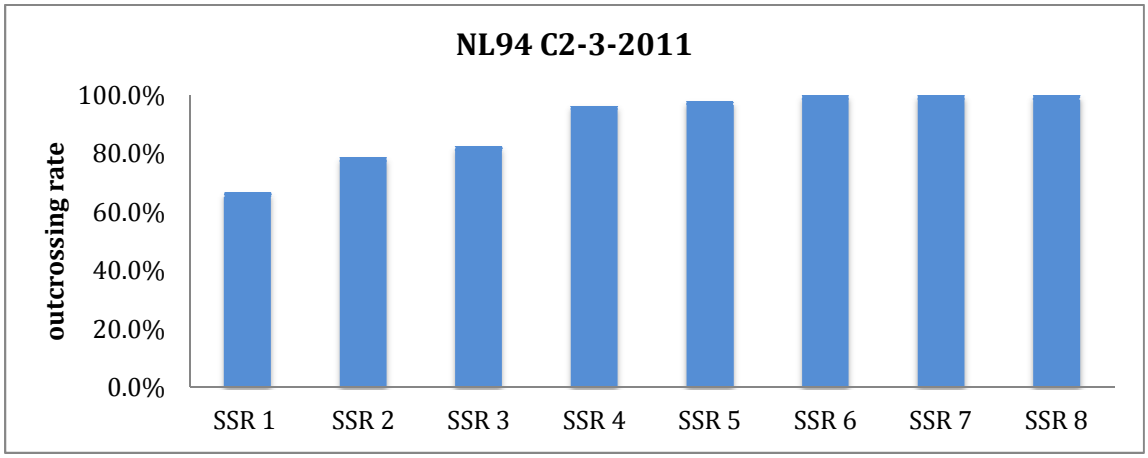


Figure 4.5 Outcrossing rates of 2010 SL93 C2-3 progeny identified with eight SSR markers

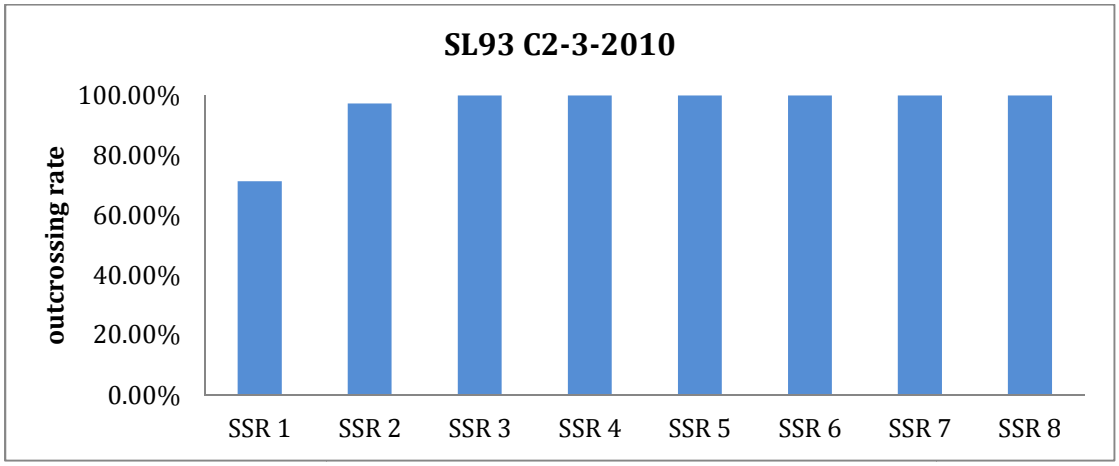


Figure 4.6 Outcrossing rates of 2011 SL93 C2-3 progeny identified with eight SSR markers

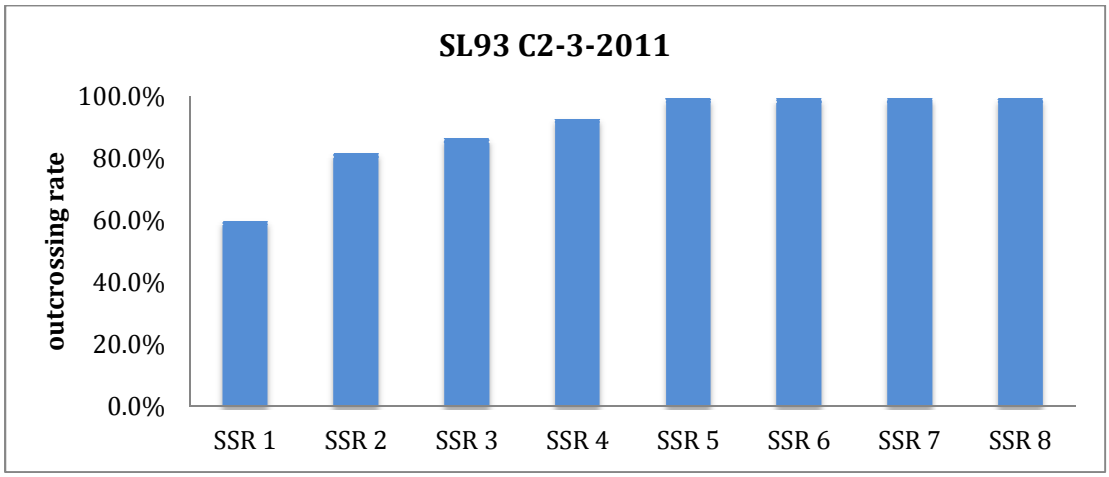


Figure 4.7 Outcrossing rates of 2010 SL93 C3 progeny identified with eight SSR markers

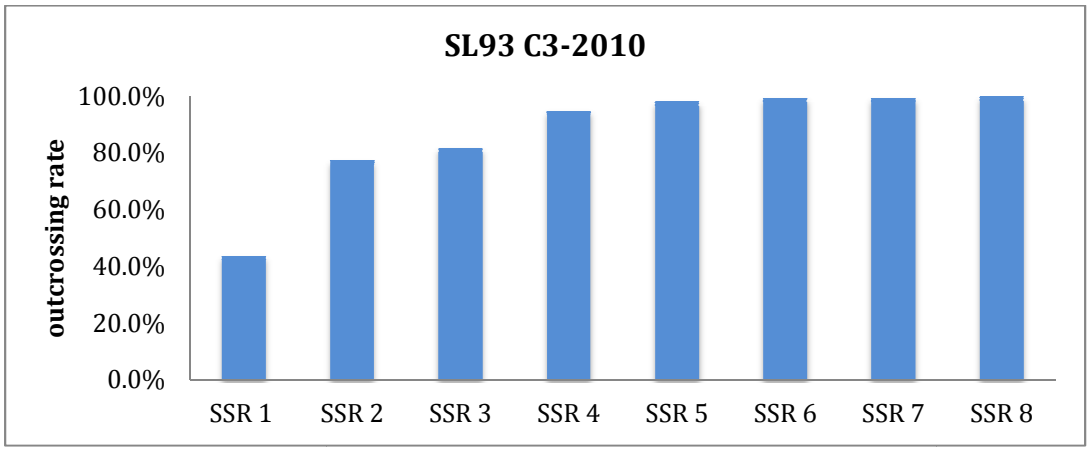


Figure 4.8 Outcrossing rates of 2011 SL93 C3 progeny identified with eight SSR markers

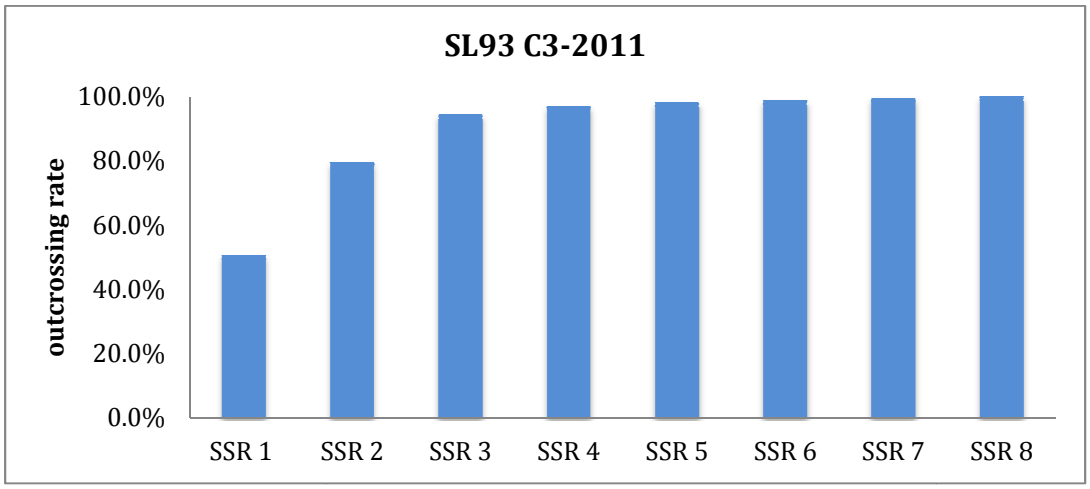


Figure 4.9 Outcrossing rates of 2010 NL94 C3 progeny identified with eight SSR markers

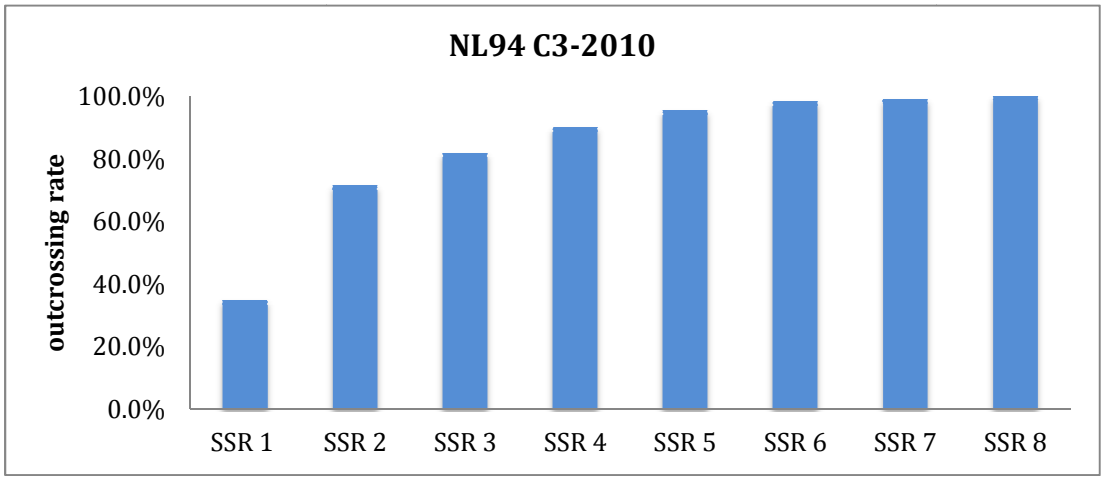


Figure 4.10 Outcrossing rates of 2011 NL94 C3 progeny identified with eight SSR markers

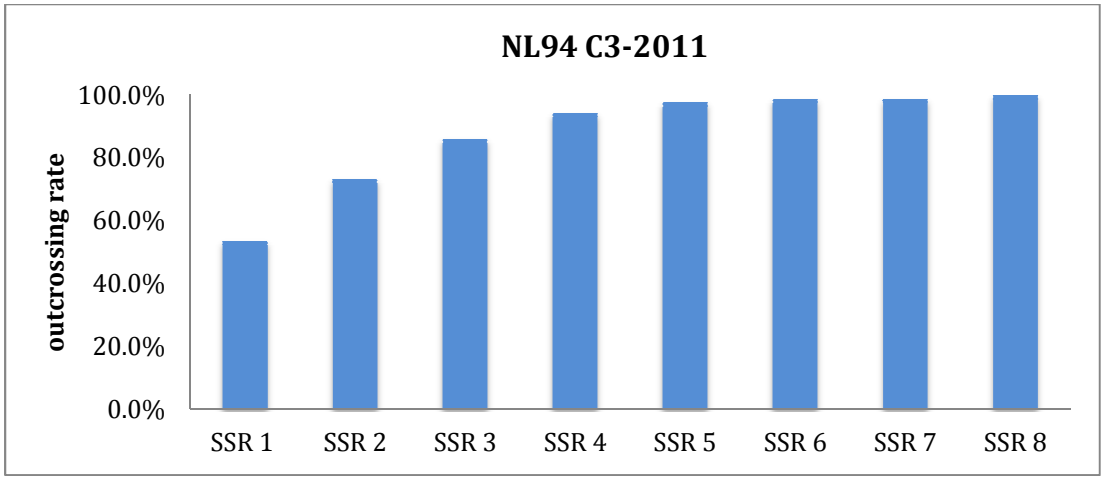


Figure 4.11 A gel image of one progeny of SL 13×6 in 2010 and one progeny of SL 4×4 in 2011 identified to be selfed with eight SSRs. M stands for standard molecular size markers with specific sizes are given on the right side of the gel. The left portion of the image is the amplification result of SL 13×6 from 2010 with eight SSRs, and the right part of image demonstrates the genotyping result of SL 4×4 from 2011 with eight SSRs. Each line under the amplified bands indicates one SSR marker amplifying one parent and one progeny DNA samples. Under each SSR, first two lanes are two-replicated parent samples and next two are progeny sample replications. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 indicate two parent replication samples; 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 represent two-replicated progeny samples.

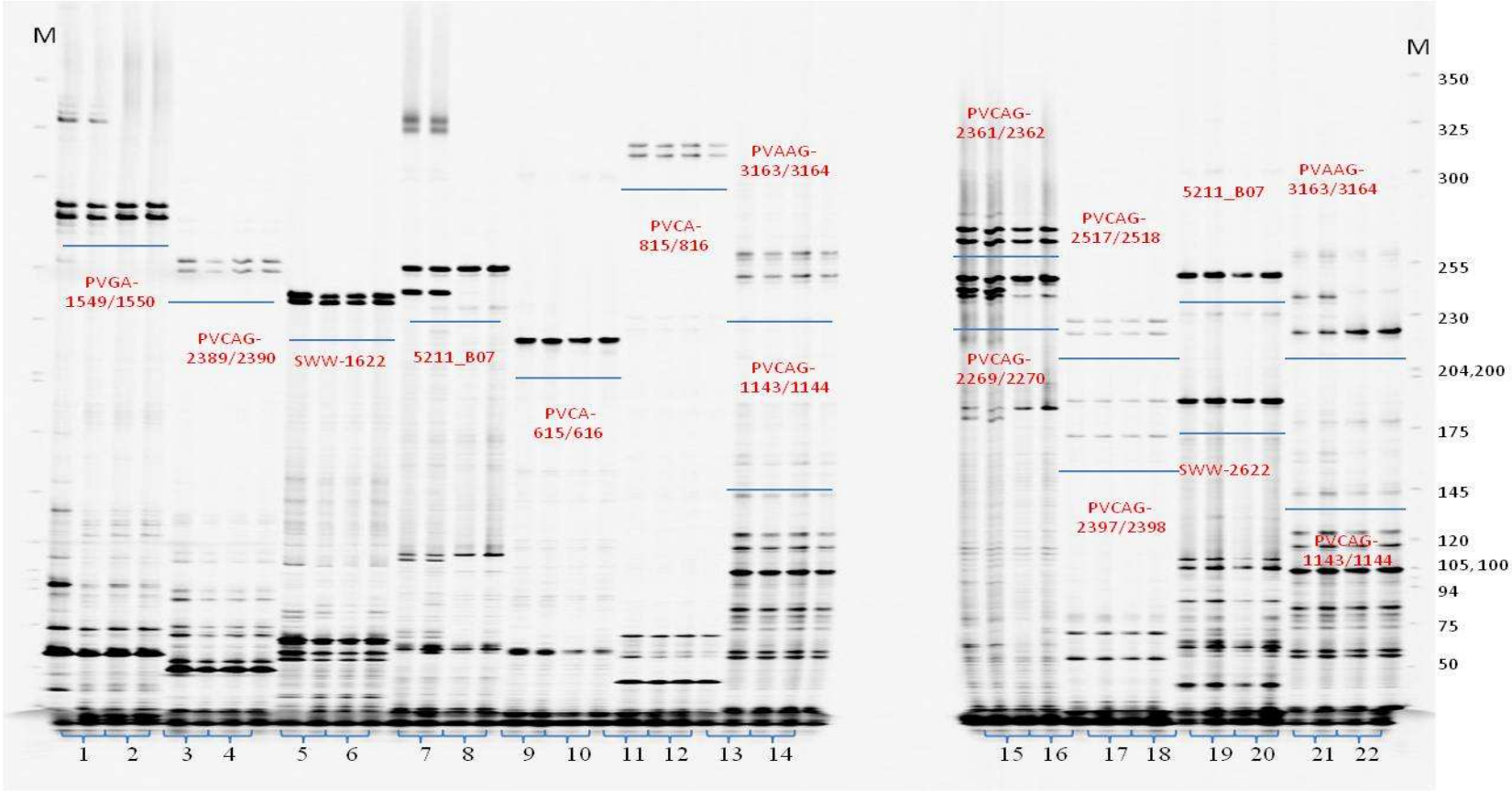


Figure 4.12 A gel image of one progeny of SL 13×6 in 2010 identified to be selfed with initial eight SSRs and to be crossed with eight additional eight SSRs. M stands for standard molecular size markers with specific sizes are given on the right side of the gel. Each line under the amplified bands indicates one SSR marker amplifying one parent and one progeny DNA samples. Under each SSR, first two lanes are two-replicated parent samples and next two are progeny sample replications. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 indicate two parent replication samples of SL 13×6; 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 represent two-replicated progeny samples of SL 13×6.

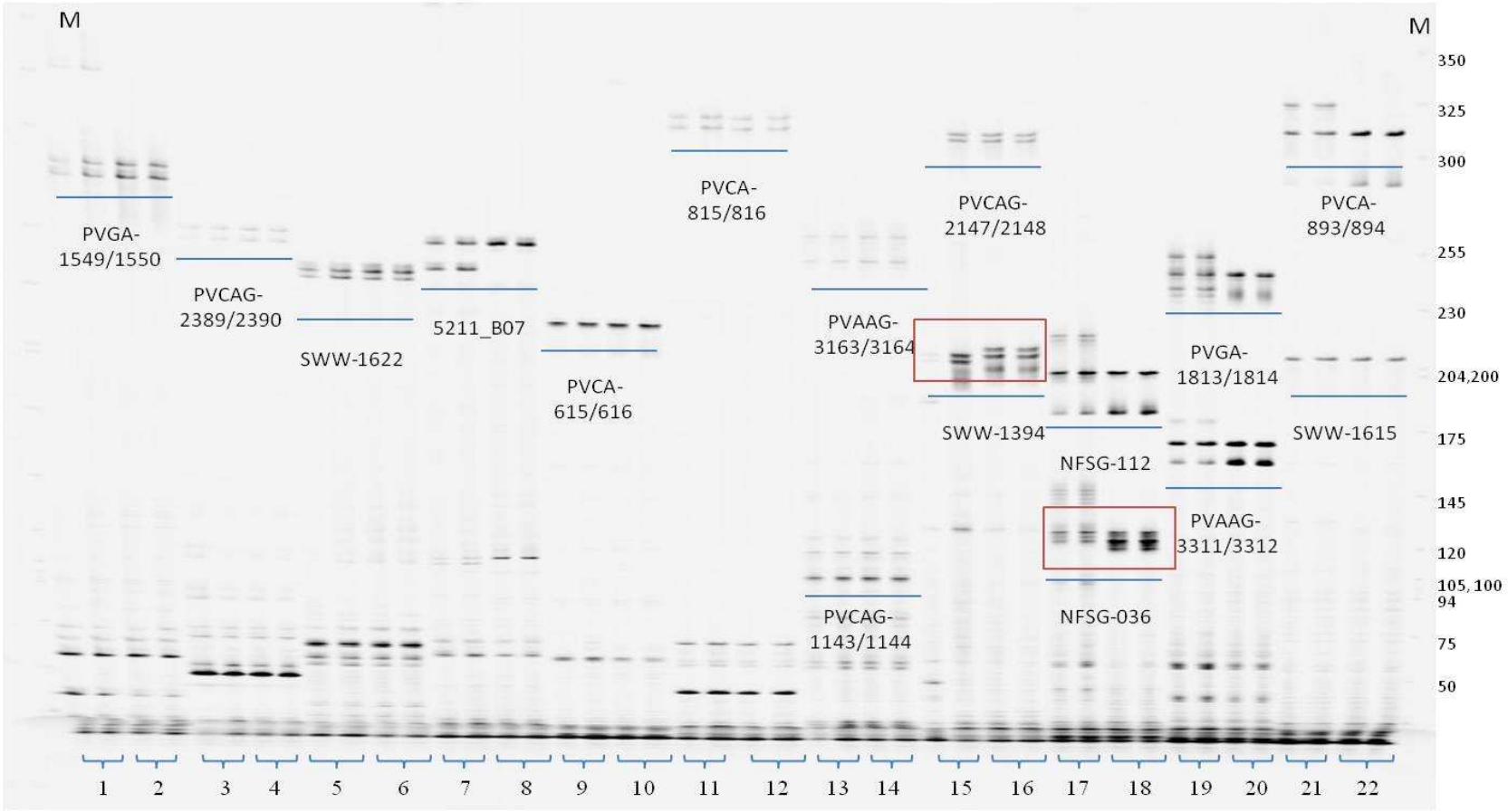
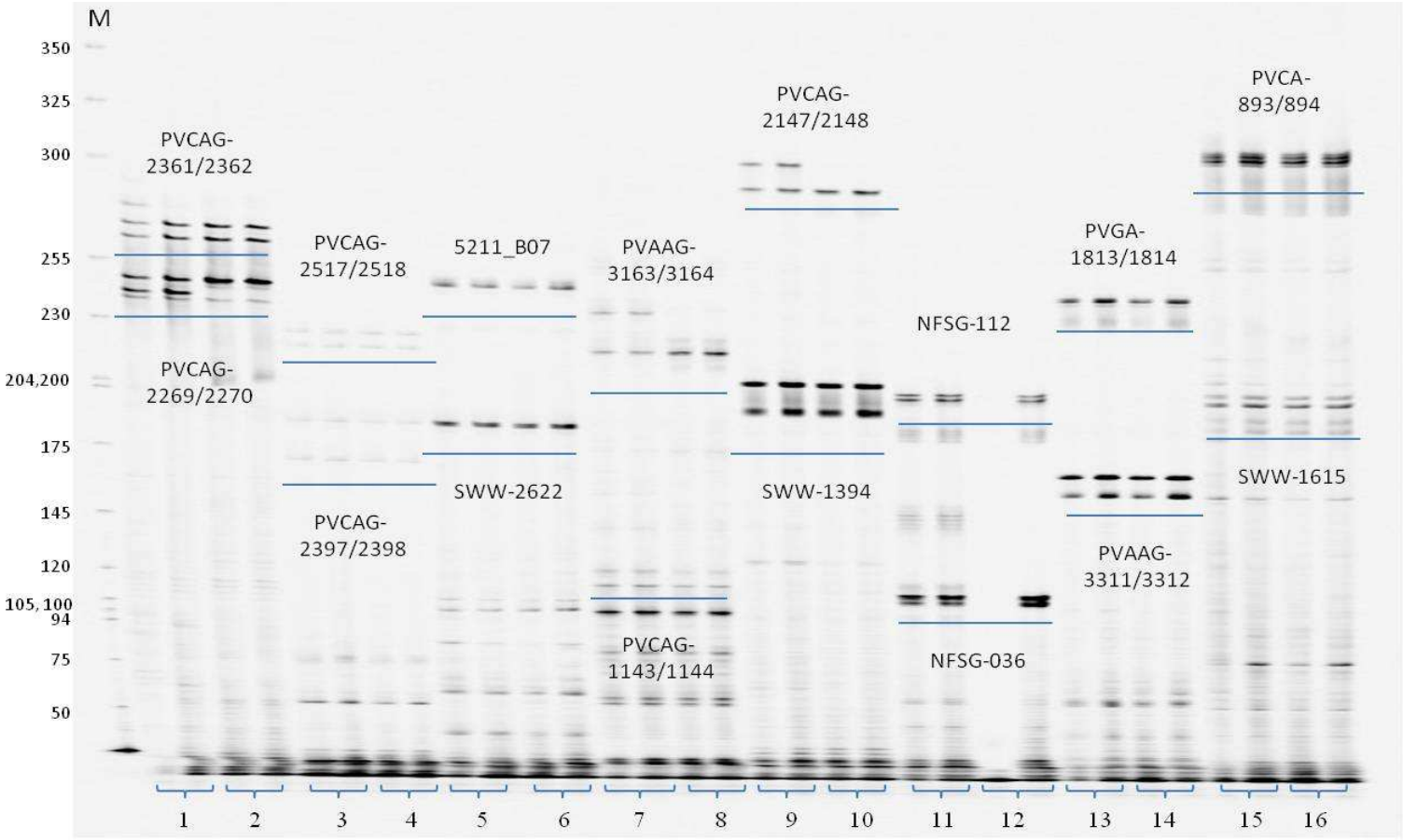


Figure 4.13 A gel image of one progeny of SL 4×4 in 2011 identified to be selfed with initial eight SSRs and to be further confirmed to be selfed progeny with eight additional eight SSRs. M stands for standard molecular size markers with specific sizes are given on the left side of the gel. Each line under the amplified bands indicates one SSR marker amplifying one parent and one progeny DNA samples. Under each SSR, first two lanes are two-replicated parent samples and next two are progeny sample replications. 1, 3, 5, 7, 9, 11, 13, 15 indicate two parent replication samples of SL 4×4; 2, 4, 6, 8, 10, 12, 14, 16 represent two-replicated progeny samples of SL 4×4.



CHAPTER V

CONCLUSION

Switchgrass has been widely recognized as a leading cellulosic perennial for bioenergy feedstock production on marginal lands. Its sexual reproduction behavior in open-pollinating environments is critical for population improvement and varietal development. The present study demonstrated lowland switchgrass plants set near 100% outcrossed seed when grown in the field and subjected to open pollination while selfed progeny were rare. Although only one, the identified selfed progeny may be valuable in inbred line development. The results should be helpful in developing a fuller understanding of the reproductive biology of switchgrass.

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