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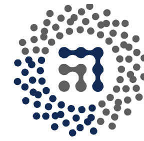

FACULTY OF SCIENCE Y TECHNOLOGY. LEIOA

BACHELOR DEGREE RESEARCH PROJECT

SIMPLE SEQUENCE REPEAT ALLELIC DIVERSITY IN ZOYSIAGRASS RELEASED FROM 1910 TO 2016

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TABLE OF CONTENTS

Introduction ………………………………………………………………………….. 1
Objectives ……………………………………………………………………………. 4
Materials and Methods ……………………………………………………………. 5
Results …………………………………………………………………………………9
Discussion and Conclusions………………………………………………………. 15
Acknowledgments ………………………………………………………………... 18
References …………………………………………………………………………... 19
Appendix …………………………………………………………………………….. 23

ABSTRACT

Selection during varietal improvement has been shown to reduce genetic diversity in several different crop species. A reduction in genetic diversity can be detrimental to future breeding efforts and increase susceptibility to biotic stresses. A better understanding of genetic diversity in Zoysiagrass would help improve breeding efforts. Over the years, microsatellite markers have been very useful to determine genetic diversity in a number of crop species. The purpose of this study is to analyze levels of genetic diversity at gene and population levels in 39 *Zoysia* cultivars (*Zoysia* spp. Willd.). 23 primer SSR markers were used to determine whether allelic diversity among these cultivars has change over a century of modern plant breeding. In this study, Zoysiagrass varieties analyzed were released between 1910 and 2016 and most of the samples were from North Carolina State University. We evaluated genetic diversity at population level and conclude that allelic diversity declined during the 1990s but recovered during the 2000s.

Our study demonstrates that the influence of science based plant breeding in Zoysiagrass has not been very successful because allelic diversity has decreased after reaching the peak in 2000s. In the future, the levels of genetic diversity should increase through future germplasm collections and the inclusion of less *Zoysia* species to create new hybrids. Additionally, SSR has proved to be useful to detect molecular variation in Zoysiagrass cultivars.

1. INTRODUCTION

Z. *japonica* know as Zoysiagrass has been cultivated in China, Japan and Korea for centuries. Zoysiagrass is an allotetraploid with 40 chromosomes (2n=4x=40) that completes a small size genome (421Mbp) (Cai et al., 2005, Harris-Shultz et al., 2012). 11 different species have been found to be part of Zoysiagrass (Arumuganathan et al., 1999 and Forbes, 1952). However, when it comes to commercial varieties *Zoysia japonica* Steud and *Zoysia matrella* (L.) Merr are mostly used in the United States for turfgrass purposes. Other species of Zoysiagrass are used to integrate new traits such as smaller leaf size and disease resistance (Engelke, 2000; Engelke and Anderson, 2003).

There is currently some question about the designation of phenotypically variable zoysiagrass into different Zoysia species' because of the ability of many of these species to readily hybridize (Kimball et al., 2013). For example, 'Crowne' and 'Palisades' are believed to be Z. japonica × Z. pacifica (Goudsw.), while 'Cavalier', 'Diamond', 'Royal', and 'Zorro' may be Z. matrella × Z. pacifica hybrids (Engelke and Anderson, 2003; U.S. Patent No. US PP11515). Kimball et al. (2013) using SSR markers suggested that five of these six cultivars were in fact hybrids and not a single Zoysia species.

Most United States germplasm was introduced in one collection trip from Asia and the relationship between private and public collections is unknown. Zoysiagrass has been a popular turfgrass in Japan since the 1700s (Tsuruta et al., 2011; Yaneshita, 2009). The first documented introduction of zoysiagrass into the US was from Korea in 1901 and the collection from Japan was brought in 1903 (USDA, 1905; Patton et al., 2017). 'Chinese common' is the earliest known zoysiagrass cultivar to be brought into the US, but there is some debate on its origin (Patton, 2009). In the early 1940s, 'Matrella' and 'Meyer' cultivars were released followed by 'Sunburst', and 'Emerald' in the 1950s. Meyer and Emerald quickly became industry standards for winter hardiness and turf quality, respectively (Patton, 2009). University of California provided the germplasm base for 'El Toro', 'DeAnza', and 'Victoria' from the USDA-NPGS (National Plant Germplasm System) and from Japan and

Korea (Engelke et al., 2003). In the 1980s, public breeding efforts in the United States were focused on improving vegetatively propagated cultivars, while private breeding programs focused on developing seeded cultivars (Engelke et al., 2003). Additionally, today turf grass breeders focus on developing improved varieties that require less maintenance.

Zoysiagrass is a warm-season turf grass with relatively high heat and drought tolerance and has disease resistance. For this reason is widely used in the Southern United States. However, it could use more cold tolerance for colder weather places in the United States. Nevertheless, the biggest problems with Zoysiagrass consist in infections by nematodes and insets.

Over the years, zoysiagrass cultivars have been improved using conventional breeding methods such as hybridization, clonal selection and mutagenesis. Since 1990, research in zoysiagrass has concentrated on improving traits such as propagation by seed, drought tolerance, cold weather tolerance, turf density, salinity tolerance, and insect resistance (Tsuruta et al., 2011). The objective in zoysiagrass production is to generate economically viable seed that have the following characteristics such as regrowth, cold hardiness, winter color retention, and disease resistance (Engelke et al., 2003).

The breeding of turf grass results in a total economic impact of $4.7 billion in the state of North Carolina. There are more than 20000 acres of turf grass cultivars, 605 Golf courses and 3.4 million dwellings (1999 North Carolina Turfgrass Survey). However, the presence of insets and pathogens, drought, fertility problems and additional problems cause the crop to spoil resulting in $276 million economic losses for producer and consumer.

To improve future cultivars genetic diversity is needed; however, intensive plant breeding usually leads to a decrease of genetic diversity. To limit the loss of genetic variability in zoysiagrass, plant collection and habitat preservation at the sources of origin are needed (Tsuruta et al., 2011). The integration of new traits is achieve by including unique alleles, not currently present in elite populations, that carry resistance against stresses that may affect production such as resistance to different diseases or weather conditions (Brown, 1983). For example, Studies on the effect of

modern plant breeding on genetic diversity in wheat have shown no loss of allele numbers or genetic diversity over time (Huang et al., 2007; Martynov et al., 2005; Donini et al., 2000).

In recent years, a number of DNA-based molecular markers have been used to determinate allelic diversity for Zoysiagrass: restriction fragment length polymorphism (RFLP; Yaneshita et al., 1997), amplified fragment length polymorphism (AFLP; Cai et al., 2004), microsatellites (Cai et al., 2005). Single repeat markers have been used to complete molecular linkage maps and to assess genetic diversity (Li et al., 2009).

In this study Simple Sequence Repeat markers (SSR) have been used to determine genetic diversity in Zoysiagrass. Previously, microsatellite markers have been very useful to determine genetic diversity because they are PCR based, they are co-dominant and they show a high level of allelic variation (Rampling et al., 2001; Roussel, 2005). Tsuruta (2005) found that SSR markers are valuable tools for identification, estimation of genetic diversity and construction of genetic linkage maps in Zoysia species. Therefore, in previous studies SSR markers have been successfully used to assess genetic diversity levels and change over time in other crop species including maize (Lu and Bernardo 2001), wheat (Roussel et al., 2005; Donini et al., 2000), barley (Hordeum vulgare L.) (Malysheva-Otto et al., 2007), oat (Avena sativa L.) (Fu et al., 2003), and Runner-Type Peanut Cultivars (Arachis hypogaea L.) (Milla-Lewis et al., 2010a; 2010b).

2. OBJETIVES

The purpose of this study is to analyze levels of genetic diversity at gene and population levels in 39 *Zoysia* cultivars. We are also going to evaluate the influence of science-based plant breeding in Zoysiagrass and assess the temporal genetic diversity. While previous studies in Zoysia spp. have evaluated genetic relationships (Kimball et al., 2013), they have not studied changes in allelic diversity over time. To achieve this, 23 primer SSR markers were used to determinate whether allelic diversity among these cultivars has change over a century of modern plant breeding. The DNA was extracted from *Zoysia* cultivars and amplified with SSR markers using PCR. Then, the DNA was separated by size in polyacrylamide gel electrophoresis. Finally, all the results were analyzed and evaluated. The Zoysiagrass varieties analyzed were released between 1910 and 2016. The source of most of the samples is North Carolina State University. The results of this study and related studies will provide plant breeders with information to increase or maintain diversity in zoysiagrass.

3. MATERIALS AND METHODS

3.1. PLANT MATERIAL AND DNA EXTRACTION

Thirty-nine zoysiagrass varieties were analyzed in this study, all of them released between 1910 and 2016 and both public and private sources were evaluated (**Table 1**). 28 of 39 seeds were obtained from North Carolina State University (NCSU) germplasm collection. The rest of the samples that could not be found in NCSU were

Table 1. List of Zoysiagrass cultivars used in the molecular diversity assessment.

Identity	Species	Breeder or Supplier	Year of release	Source	Period
Chinese Common	*Z. japonica*	Introduction from China	1910	NCSU	1910s
Matrella	*Z. matrella*	Alabama Agric. Experiment Station	1941	NCSU	1940s
Meyer	*Z. japonica*	United States Department of Agriculture	1951	NCSU	1950s
Emerald	*Z. japonica x Z. pacifica*	United States Department of Agriculture	1955	NCSU	1950s
Midwest	*Z. japonica*	Indiana Agricultural Experiment Station	1963	NCSU	1960s
El Toro	*Z. japonica*	University of California, Riverside, CA	1986	NCSU	1980s
Belair	*Z. japonica*	United States Department of Agriculture	1987	NCSU	1980s
Cashmere	*Z. matrella*	Pursley Turf Farms, Palmetto, FL	1989	NCSU	1980s
GN-Z	*Z. japonica*	Greg Norman Turf Company, Jupiter, FL	1989	Purdue	1980s
DeAnza	*Z. japonica*	West Coast Turf, Stevinson, CA	1995	NCSU	1990s
Victoria	*Z. japonica*	West Cost Turf, Stevinson, CA	1995	NCSU	1990s
Cavalier	*Z. matrella*	Texas A&M University, Dallas, TX	1996	NCSU	1990s
Crowne	*Z. japonica*	Texas A&M University, Dallas, TX	1996	NCSU	1990s
Diamond	*Z. matrella*	Texas A&M University, Dallas, TX	1996	NCSU	1990s
JaMur	*Z. japonica*	Bladerunner Farms, Inc., Poteet, TX	1996	NCSU	1990s
Palisades	*Z. japonica*	Texas A&M University, Dallas, TX	1996	NCSU	1990s
Zeon	*Z. matrella*	Bladerunner Farms, Inc., Poteet, TX	1996	Purdue	1990s
Empire	*Z. japonica*	Sod Solutions, Inc., Mt. Pleasant, SC	1999	NCSU	1990s
Compadre	*Z. japonica*	Seed Research of Oregon, Corvallis, OR	2000	NCSU	2000s
Empress	*Z. japonica*	Sod Solutions, Inc., Mt. Pleasant, SC	2000	NCSU	2000s
Zenith	*Z. japonica*	Patten Seed Co, Lakeland, GA	2000	NCSU	2000s
Royal	*Z. matrella*	Texas A&M University, Dallas, TX	2001	Purdue	2000s
Zorro	*Z. matrella*	Texas A&M University, Dallas, TX	2001	NCSU	2000s
Himeno	*Z. japonica*	Winrock Grass Farm, Little Rock, AR	2002	NCSU	2000s
PristineFlora	*Z. matrella*	University of Florida, Belle Glade, FL	2005	Purdue	2000s
UltimateFlora	*Z. japonica*	University of Florida, Belle Glade, FL	2005	Purdue	2000s
Carrizo	*Z. japonica*	Bladerunner Farms, Inc., Poteet, TX	2006	Purdue	2000s
Y2	*Z. japonica*	Bladerunner Farms, Inc., Poteet, TX	2006	NCSU	2000s
Shadowturf	*Z. matrella*	Ivey Gardens Greenhouses, Lubbock, TX	2007	NCSU	2000s
Marion	*Z. japonica*	Heritage Turf, Inc., Midway, AL	2008	NCSU	2000s
Rollmaster	*Z. matrella*	Winrock Grass Farm, Little Rock, AR	2008	NCSU	2000s
Serene	*Z. japonica*	Heritage Turf, Inc., Midway, AL	2008	NCSU	2000s
Southern Gem	*Z. japonica*	Heritage Turf, Inc., Midway, AL	2008	NCSU	2000s
Geo	*Z. matrella*	Sod Solutions, Inc., Mt. Pleasant, SC	2010	NCSU	2010s
Innovation	*Z. japonica*	Sod Solutions, Inc., Mt. Pleasant, SC	2010	SS	2010s
Chisholm	*Z. japonica*	Texas A&M University, Dallas, TX	2012	Purdue	2010s
Cutlass	*Z. japonica*	Bladerunner Farms, Inc., Poteet, TX	2013	UGA	2010s
L1F	*Z. matrella*	Bladerunner Farms, Inc., Poteet, TX	2014	NCSU	2010s
Lowrider 2	*Z. japonica*	Bladerunner Farms, Inc., Poteet, TX	2016	UGA	2010s

obtained from other breeding programs assess in **Table 1**. The cultivars were organized according to the year of release and were separated in groups according to the decade of release (1910s, 1940s, 1950s, 1960, 1980s, 1990s, 2000s and 2010s).

Zoysiagrass cultivars were grown and maintained at North Carolina State University Crop Science Greenhouse for 5 weeks previous to extraction. Then, 8 young fresh leaves were collected and keep on ice until DNA extraction. It is important to start with the DNA extraction as soon as possible to avoid contamination and DNA degradation. DNA was extracted according to CTAB (cetyl trimethyl ammonium bromide) protocol (Stein et al. 2001) except that in this study a Fast Prep FP120 machine (Qbiogene, Irvine, CA) was used to break the leave tissue.

After the DNA was extracted quality was analyzed using spectrophotometric quantification method [Hoefer DQ 300 fluorometer (Hoefer Inc., San Francisco, CA)]. Finally, all DNA samples were diluted to a final concentration of 10 ng/µL and store at 4ºC. To determinate that the DNA extraction was successful a quality agarose gel was run.

3.2. PCR WITH SSR MARKERS

The 23 primer pair Single Sequence Repeat markers used in this study are listed in **Table 2**. The SSR primer pairs were previously developed in Guo et al. (2012), Ma et al. (2007) and Li et al. (2009). The DNA sequences of the primer pair listed in **Table 2** are shown in the appendix. Eurofins Scientific (Mebane, NC) delivered the primers for the study. The PCR for SSR markers was performed using fluorescent M13-tail labeling method of Schuelke (2000). This method involves adding a universal M13 (6-Fam, Hex and Ned) primer labeled with IRD-700 or IRD-800 (LICOR Biosciences, Lincoln, NE). IRD-700 and IRD-800 labels allow fluorescent primer extension and it is used to visualize the DNA in the gel electrophoresis.

The PCR mix contained 1.26 µL H2O, 1µL 10X buffer (New England Biolabs, Ipswich, MA) 4µL betaine (2.5M), 0.8µL dNTP, 0.46µL $MgCl_2$, 0.3µL forward primer, 0.4µL reverse primer, 0.2µL m13 fluorescence (700/800) and 0.08µL taq DNA polymerase in each PCR reaction in a well. The 8.5µL of the PCR mix were added to the 15 ng of sample DNA. The solution was centrifuged briefly and place in

the PCR machine. The following termocycling conditions were used: 94ºC for 5 minutes, then 30 cycles of denaturing 94ºC for 30 s, Tm (specific annealing temperature depending on the Tm-rev of the primers used) for 45 s and 72ºC for 42 s, and a final extension at 72ºC for 5 minutes.

3.3. GEL ELECTROPHORESIS

Following the PCR amplification the DNA products were separated in polyacrylamide gel electrophoresis (PAGE). This is a technique used for the detection and separation of DNA molecules. An electric field is applied to a acrilamide gel matrix, and within the gel, charge particles will migrate and separate based on size. The equipment used was LI-COR 4300 DNA Analyzer Sequencer (LI-COR Bioscience, Lincoln, NE) using 12% polyacrylamide gels [7M ultrapure urea, 0.8x TBE buffer and 12% LongRanger acrylamide (BioWhittaker Molecular Application, Rockland, ME)]. To achieve that, we added 10 µL of formide loading dye to each well (95% deionized formamide, 20 mmol ethylenediaminetetraacetic acid (EDTA) and 0.8 mg mL^{-1} bromophenol blue) and denatured in the PCR equipment heating at 94ºC for 3 minutes and a final extension at 4ºC. Then, the samples were kept on ice until loading the gel. SSR marker amplifications were separated in the gels by size and the polymorphic bands were scored using the bioinformatics program Quantar 1.0 (Keygene Products B.V., Wageningen, The Netherlands).

3.4. DATA ANALYSIS

Total number of alleles and the percentage of rare alleles (frequency < 0.05) comparing to all alleles were determinate for each primer pair. The total number of alleles was also calculated for each time period. The number of cultivars for each decade had an effect in the quality and result of allele variability.

Values of genetic similarity were measured between the cultivars in the same decade and the samples in other decades (Dice, 1945). The software used was NTSYS-PC version 2.2 (Exeter Software, Setauket, NY) (Rohlf, 2000). Subsequently, average genetic similarities were calculated for each decade of release to test for significant differences between each pair of groups.

The genetic relationship among cultivars was summarized in terms of cluster and principal coordinate analysis (PCO) based on NTSYS-PC version 2.2 program. To perform 2D-principal coordinate analysis Dcenter and Eigen option from NTSYS program was used. The similarity dendogram was obtained using Unweighted Pair Group Method in NTSYS (Sneath and Sokal, 1973).

4. RESULTS

4.1. MICROSATELLITE POLYMORPHISM

23 SSR primer pairs amplified a total of 200 different alleles with an average of 8.69 alleles per primer pair (**Table 2**). B03G12 (**Figure 1**) and B02C06 were the primer pairs that identified more alleles with 15 alleles each being highly variable. On the other side, GB-ZJM-07 detected only 4 alleles.

From 200 alleles identified 43 were considered to be rare (frequency < 0.05). Consequently, 21.5% of all alleles were rare alleles. For instance, B02G17, B03G12 and B02C06 had 5, 6 and 7 rare alleles respectively.

Figure 1 shows the amplified DNA for different cultivars of Zoysiagrass using B03G12 SSR marker. On both sides of the gel molecular weight marker is loaded. Moreover, each of the lanes represents a different cultivar from **Table 1** and 'chinese common' and 'Midwest' are used as control samples. Finally, all the bands observed in each lane represent allelic polymorphism for each Zoysiagrass cultivar.

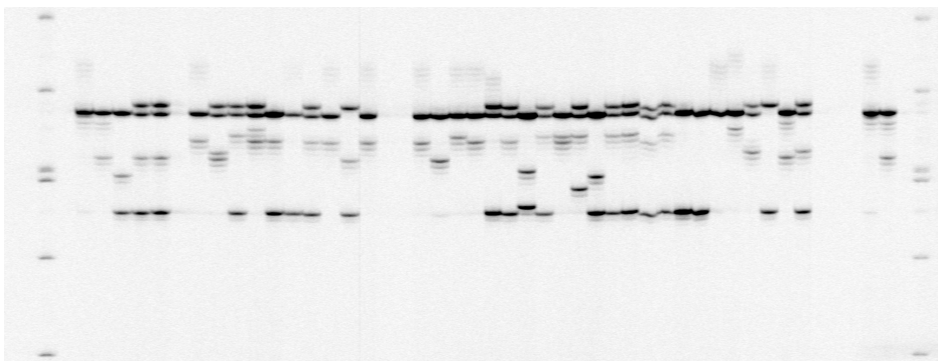

Figure 1. B03G12 SSR primer pair gel electrophoresis image showing polymorphic bands.

4.2. CHANGE IN ALLELIC COUNTS

The number of alleles for cultivars released during 2010s was higher comparing to the ones released in 1910s in all loci (**Table 2**). In the 1910s the highest number of alleles measured is three. For all loci as a group, allelic counts have increased from 910s to 2000s. However, for most of the samples the peak of allelic counts was during 2000s and on 21 out of 23 loci (91.3%) allelic counts decreased for the cultivars from 2010s (**Figure 2a**). The other two loci (8.69%) have the same allelic

Table 2. Simple sequence repeat (SSR) primer pair list used for evaluating Zoysiagrass cultivars and corresponding results of allelic diversity.

Primer pair	Total No. of alleles	No. of rare alleles†	Allelic counts by breeding decade							
			1910s (1)	1940s (1)	1950s (2)	1960s (1)	1980s (4)	1990s (9)	2000s (15)	2010s (6)
A02O06	7	0	1	3	2	3	6	7	7	6
B01N07	8	2	1	1	3	2	5	4	7	6
B02G17	12	5	2	2	4	2	6	5	9	7
B03C24	6	0	1	1	2	1	4	3	6	6
B03G12	15	6	2	3	4	3	9	6	12	8
B03H23	8	0	2	2	3	3	5	7	8	4
B03O03A	11	2	1	2	3	4	6	5	10	6
B04C05	7	2	2	2	3	3	3	5	6	4
B06A14	9	2	3	1	4	4	5	6	7	6
B07I05	10	1	2	2	2	3	6	5	9	6
B08J05B	9	2	1	1	1	2	6	6	7	6
B09O08	10	0	3	2	4	3	7	8	10	10
B09J13	9	1	1	4	2	2	6	6	7	6
C03L24	5	0	1	2	2	2	2	2	5	4
B02D15	7	1	3	3	4	1	5	5	7	5
B02G05	13	3	1	2	4	2	6	6	10	9
B01P03	7	3	1	1	2	2	5	6	6	5
B02C06	15	7	1	3	2	2	4	7	11	7
D02B12	5	1	1	1	2	1	3	2	5	4
GB-ZJM-07	4	1	1	1	1	1	2	3	4	2
ZA01C06	7	1	1	1	3	2	4	6	7	4
ZA01C15	11	1	2	2	3	2	5	5	7	6
ZA01M20	5	2	1	1	3	2	4	3	4	3
Observed total	200	43	35	43	63	52	114	118	171	130

† refers to alleles present at a frequency < 0.05

count. The average of alleles in this decade is 7.43 the highest being 12 (B03G12, **Figure 1**) and the lowest 4 (GB-ZJM-07 and ZA01M20). Meanwhile, in the 1980s B03G12 scores 9 alleles and the variability keeps increasing until reaching the highest point during the 2000s. It is also important to note that the numbers of cultivars analyzed in 2000s are 15 comparing to a lower number in all the other decades. The number of cultivars released before 1980 were: one in 1910s, 1940s and 1960s and two in 1950s (**Figure 2b**). Additionally, these results were not very reliable to make more remarks.

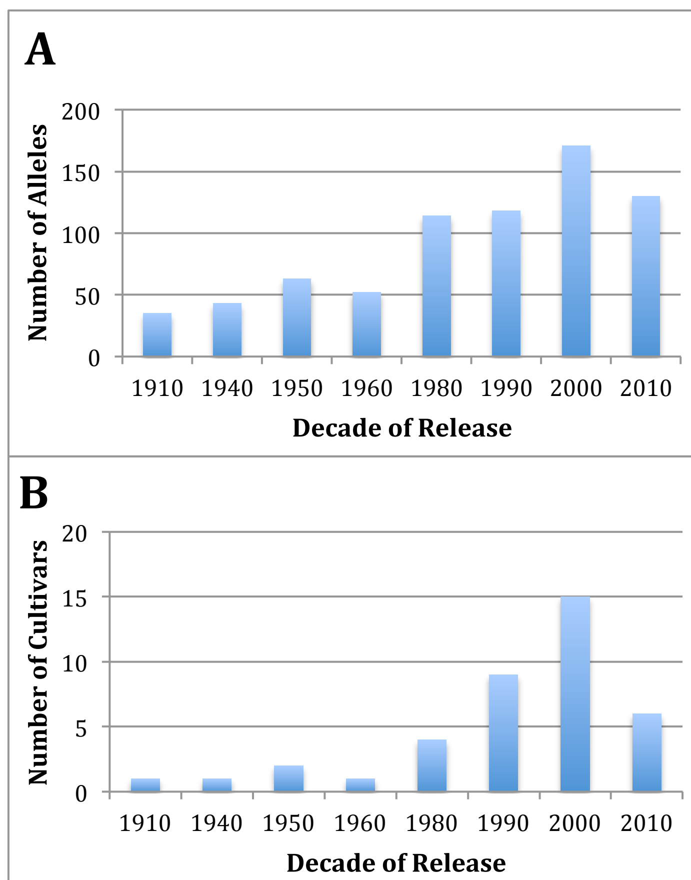

Figure 2. Total number of alleles identified with 23 primer pairs in 39 Zoysiagrass cultivars per decade of release (A). Number of cultivars release in each period (B).

4.3. GENETIC SIMILARITY

Diversity at population level was measured using Dice's method (Dice 1945). Similarity was determinate between cultivars in the same period and samples from one period against another period (**Table 3**). The cultivars released in the 1990s were very similar to each other (S_{ij} = 0.51). On the other side, the 1940s cultivars were not similar to the samples in the 1960s as observed in S_{ij} value 0.22. However, the results from the 1910s to the 1960s were not reliable due to a small number of cultivars and were not used to analyze the results. The cultivars released in the period of 1980s showed high diversity within the same decade (0.35).

Table 3. Average genetic similarity values (S_{ij}) within and between Zoysia cultivars released by decade based on 23 Zoysia simple sequence repeat markets

	1910 †	1940 †	1950	1960 †	1980	1990	2000	2010
1910	-							
1940	0.5	-						
1950	0.4	0.4	0.46					
1960	0.26	0.22	0.36	-				
1980	0.29	0.3	0.43	0.45	0.35			
1990	0.23	0.31	0.43	0.49	0.46	0.51		
2000	0.28	0.36	0.42	0.39	0.42	0.43	0.44	
2010	0.32	0.35	0.41	0.46	0.43	0.4	0.41	0.47

† Only one cultivar per decade

4.4. CULTIVAR GROUPING

Cluster analysis of the Dice genetic similarity values was performed to generate a UPGMA dendrogram to determinate the results of overall genetic relatedness among Zoysiagrass cultivars (**Figure 3**). Four main clusters are observed in the dendrogram shown in different colors. The second and third clusters have the highest number of cultivars. The cultivars marked with a red rectangle are varieties of Zoysiagrass *matrella* species. The rest of the samples are *japonica* varieties. The *matrella* cultivar was group in the first cluster isolated from the rest of the *matrellas* that are mostly in the second cluster. Most of the cultivars from the 2000s (10 from 15 cultivars) are in the second cluster resulting in lower diversity. However, the last cluster is completed with Himeno and Ultimate Flora varieties from the 2000s period. This cluster is isolated from the rest of the clusters showing no similarity to the rest of the samples and has just two cultivars. The releases from 1980s show the highest diversity being the cultivars separated in three of the clusters. El toro, Jamur, Crowne, Empire and

Zenith share a most recent common ancestor. In the decades that there were just one or two cultivars we could not make an association (1910s, 1940s, 1950s and 1960s). The dendrogram accomplished in Kimball et al. (2013) produced similar clustering patterns separating Z. japonica and Z. matrella into distinct clusters.

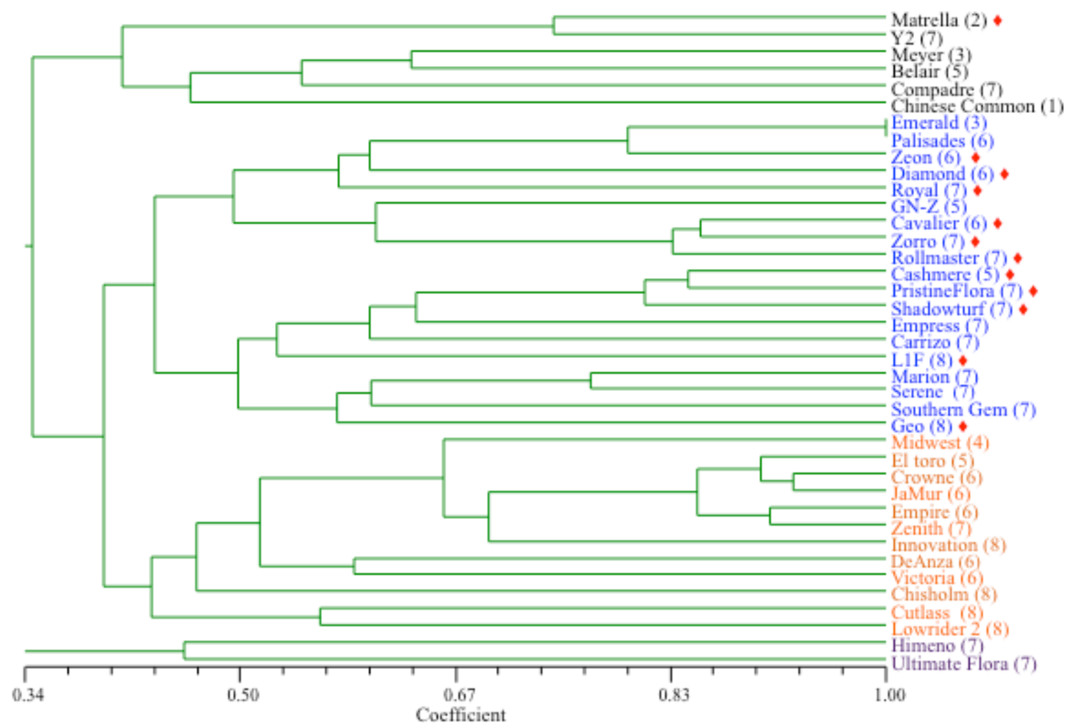

Figure 3. Single Sequence Repeat (SSR) based unweighted pair group method with arithmetic averaging (UPGMA) dendrogram of 39 Zoysiagrass cultivars released between 1910 and 2016. Different colors represent different clusters. Number in parenthesis represents the decade of release (1=1910s, 2=1940s, 3=1950s, 4=1960s, 5=1980, 6= 1990s, 7=2000s and 8= 2010s)

4.5. PRINCIPAL COORDINATE ANALYSIS

The last statistical analysis perform in this study was the 2D- Principal Component (PCO). Four 4 principal components showed a high diversity (**Figure 4**). In the graphic the dots from the same period are connected using the most isolated points that create the biggest component. Some overlap of the polygons was observed. 2000s cultivars were the most diverse ones observing the biggest area for the polygon and overlap with all other clusters (**Figure 4**, yellow). Then, the most diverse period is the 1990s followed with the cultivars in 2010s observing a smaller

polygon size indicating less variability. The periods 1910, 1940 and 1960 are composed by one cultivar consequently a polygon is not created. There are two cultivars (Meyer and Emerald) in the 1950s creating a line in the 2D principal component. In conclusion, the cultivars released between 1910 and 1960 do not provide information in the principal component analysis.

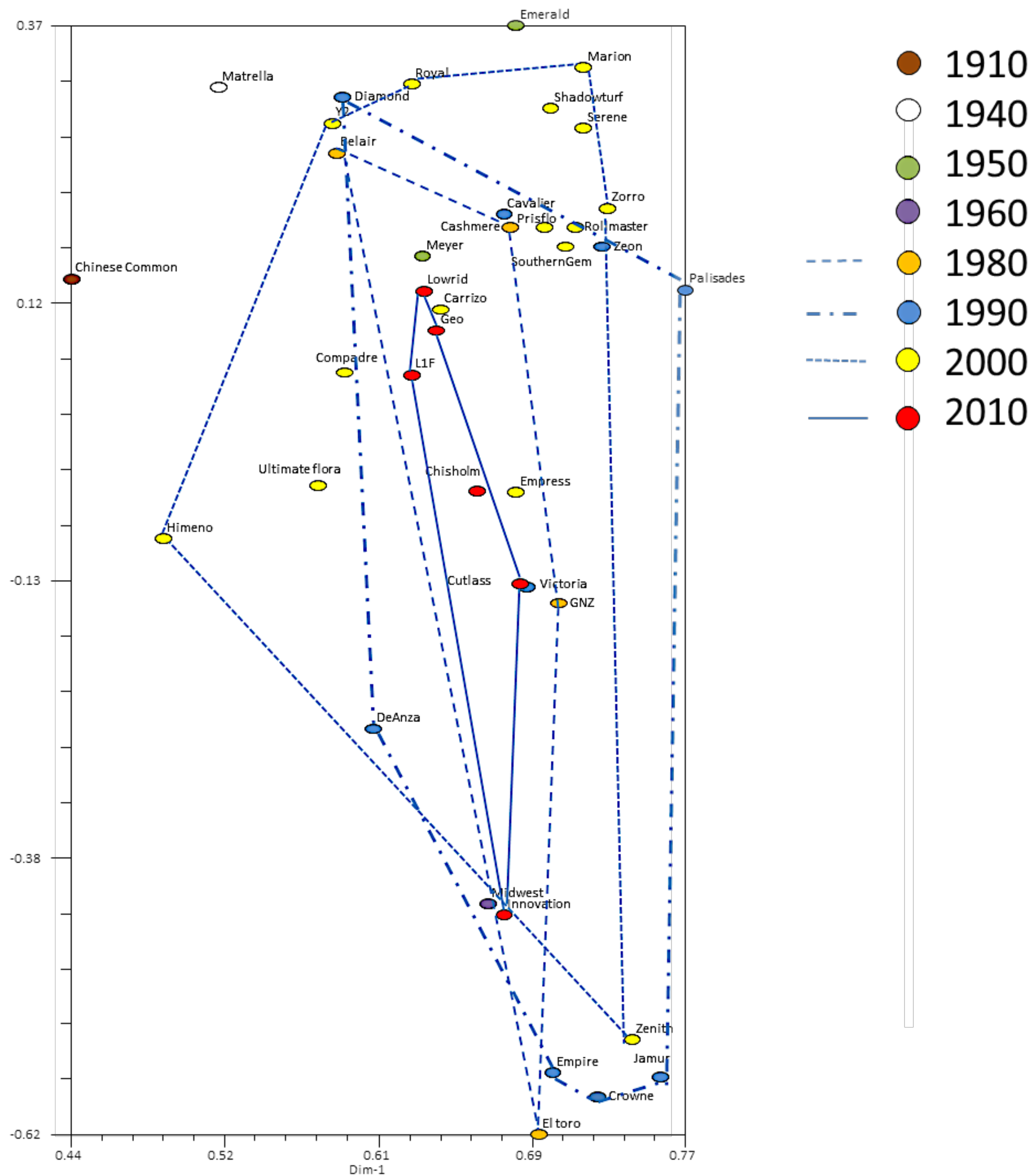

Figure 4. 2D-Principal component plot of 39 Zoysiagrass cultivars for two principal components estimated with 23 SSR primer pairs. Convex hulls around the extremes of respectively decades represent the diversity of each of those time periods.

5. DISCUSSION AND CONCLUSSIONS

The main purpose of this study was to assess whether modern plant breeding has had an impact on levels of genetic diversity in zoysiagrass cultivars released in the last century (1916-2010). To achieve this, we analyze levels of genetic diversity at gene and population levels in 39 commercial *Zoysia* cultivars. 23 primer SSR markers were used to determinate that the genetic diversity among cultivars have change over a century of modern plant breeding reducing levels of genetic diversity. 23 SSR amplified a total of 200 different alleles with an average of 8.69 alleles per primer pair. We have report a large number of alleles showing genetic diversity in Zoysiagrass collection. Consequently, Simple Sequence Repeat markers could be useful to assess molecular variation in the future.

We evaluated genetic diversity at population level and conclude that the number allelic counts for all locis have increased from 1910s to 2000s (**Table 2**). However, the peak of allelic counts was during 2000s followed by decrease of allelic counts for 2010s cultivars. The increase of allelic counts reflects a larger number of ancestors contributing to the cultivars released in 1980s and 2000s. Determining the time periods with greater amounts of allelic diversity will be useful for plant breeders to monitor their efforts to ensure that genetic diversity is maintained or increased.

Cultivars from the pre-1980s provided the genetic base for zoysiagrass breeding in the United States. Early cultivars were able to provide diverse sets of alleles, given that they were developed from plant introductions. Cultivars, such as Chinese Common and Emerald, provided a diverse set of alleles that were later distributed into several different population groups. Hybridization between species enables significant amount of genetic exchange between species as discussed by Kimball et al. (2013). While Z. *japonica* and Z. *matrella* are the two species most commonly used, other species such as Z. *minima* (Colenso) Zotov, Z. *machrostachya* Franch & Say, Z. *pacifica*, and Z. *sinica* Hance have been utilized for trait improvement (Engelke, 2000; Tsuruta et al., 2011).

As observed in the similarity analysis (**Table 3** and **Figure 3**) the cultivars from 1980s are very variable (S_{ij} = 0.35). Consequently, it would be a good option to follow the breeding methods of this period. On the other side, the cultivars released

in the 1990s were very similar to each other (0.51) consequence of less effective breeding programs.

Since the 1990s, breeding efforts in zoysiagrass have increased leading to the improvement of traits such as cold and drought tolerance, as well as pest resistance (Patton, 2009). These efforts might have contributed to the significant increase in allele numbers observed in the 2000s. Cultivars, such as Himeno (U.S. Patent No. US 20020092083), discovered in a collection expedition to Yaku Island (Japan), are sources of new genetic diversity in zoysiagrass. The results in 2D- Principal Component (**Figure 4**) showed that diversity has increase throughout breeding periods until reaching the peak in 2000s. Then, the most diverse period is the 1990s followed with the cultivars in 2010s observing a smaller polygon size indicating less variability. In conclusion, the genetic diversity peak is reached in 2000s then decreasing the diversity for Zoysiagrass in 2010. Nevertheless, given that this decade is not yet over, special consideration should be taken when making comparisons between the 2000s and 2010s. It is still possible to have an increase in genetic diversity levels comparable to the previous decade during the late 2010s.

It is also important to note that the numbers of cultivars analyzed in 2000s are 15 comparing to a lower number in all the other decades. In the period previous to 1980s five cultivars were released in total. Additionally, these results were not very reliable to make more remarks. Due to the low number of cultivars between 1910 and 1960, in the future the principal component analysis should be performed grouping them all in the same group. These results would show a higher genetic diversity if the cultivars are group together. Consequently, all the statistical analysis performed in this study would have had more productive results if all the cultivars previous to 1980 were grouped together.

New sources of genetic variation will be useful in zoysiagrass for continued improvement. The nine underutilized species of zoysiagrass and wild populations are potential sources for new traits. Hybridization between Zoysia spp. will also create new and useful combinations of alleles.

When it comes to breeding programs, public and private breeding programs often differ in their germplasm sources and sharing of plant material. Private programs are

much more restricted and will only share germplasm within their company, unless trade agreements are reached with other programs (Morris et al., 2006). Therefore, in private programs a decrease in diversity is possible if new sources of germplasm are not utilized or shared between programs.

We can demonstrate that the influence of science based plant breeding in Zoysiagrass has not been very successful because allelic diversity has decreased in the last decade of breeding. Scientist will have to improve the diversity in Zoysiagrass for smarter plant breeding results. To continue to improve and increase the diversity of US zoysiagrass, collection expeditions to the source of origin, the Pacific Rim, are needed (Tsuruta et al., 2011). With continued new sources of germplasm and hybridization of existing germplasm, zoysiagrass breeders will have the necessary genetic diversity for continued improvement. The conservation of genetic variation in Zoysiagrass is very important. By increasing the levels of genetic diversity the ability to respond to different weather changes, pests and diseases improves. Additionally, important agronomic traits such as yield would increase with the correct genetic improvement. In the future, the levels of genetic diversity should increase to recover the diversity from the cultivars of 1980s and 2000s.

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8. APPENDIX

Table 4. Zoysia SSR primers sequence list.

SSR markers	Repeat motif	Forward primer sequence with m-13 tail (5'-3')	Reverse primer	Size	Amplification	Rice Chromo	Source
a02o06	(TG)14(CG)7	CACGACGTTGTAAAACGACGATTGAAGCTCATGTCTATGTG	GCAATAAACAACTCTCTTCCTC	156	G	ch8	Li et al 2009
b01n07	(GA)14	CACGACGTTGTAAAACGACTGGAACCAGAGACTATCCTATC	ACACATGGTGATGGCTTC	130	G	ch6	Li et al 2009
b02g17	(TC)16	CACGACGTTGTAAAACGACTCAACGAGAGGAGAGCAG	CACATCCATGTGCTTCCT	226	G	ch12	Li et al 2009
b03c24	(GA)30..(GA)9	CACGACGTTGTAAAACGACGACTGCTGGATATGCCTG	CTACGACCTTCTCTCGTCTG	171	G	ch1	Li et al 2009
b03g12	(GA)15	CACGACGTTGTAAAACGACGTTCACAGTTCAAGAAAGCC	TTGCTCTCACTCCAAAGGA	181	G	ch4	Li et al 2009
b03h23	(TC)18	CACGACGTTGTAAAACGACAAGACCATTGTAGGCTCAAA	CCCTGGCCTTAAACAGTT	137	G	ch8	Li et al 2009
b03o03a	(GA)10	CACGACGTTGTAAAACGACTGCAACAGGTAGCTGGTAG	GCATCAGCAGGTAGATTCTC	232	G	ch1	Li et al 2009
b04c05	(GA)13	CACGACGTTGTAAAACGACCCTCCTTTTCCAATCGAT	CCTCTCCCTTCCTTCTCTT	100	G	ch2	Li et al 2009
b06a14	(GA)18	CACGACGTTGTAAAACGACACTCGTTTTCTGCACGATAT	GAAATATATCGTACGTGCTGG	112	G	ch11	Li et al 2009
b07i05	(GA)24	CACGACGTTGTAAAACGACATGGAATTAATCTTCTCCCTG	GTTGCCTCTTTCATACTTGTG	112	G	ch3	Li et al 2009
b08j05b	(TC)6..(TC)15	CACGACGTTGTAAAACGACTGACCGTTGTAGCCTAGC	GAACAAGAACGGGTAGGG	125	G	ch1	Li et al 2009
b09o08	(TC)17	CACGACGTTGTAAAACGACATCTGGATCAGCTTTCAAGA	AAAGGTCGCTAGATTGATCA	156	G	ch2	Li et al 2009
b09j13	(GA)14	CACGACGTTGTAAAACGACCAGCAACATCAGACAAACC	GAGAATACCTTTCTGCCTTTC	115	G	ch5	Li et al 2009
c03l24	(ttc)8	CACGACGTTGTAAAACGACCAGCCAGCACATAGGATC	GAGTGAGACCTGCACGAG	249	S	ch6	Li et al 2009
b02d15	(GA)17	CACGACGTTGTAAAACGACCGAGCGAAGTCAAAGAAG	TCGTTTCTTCTTGAGTCTCTG	121	N	ch3	Li et al 2009
b02g05	(GA)20	CACGACGTTGTAAAACGACGGAGCATATATGGATGCATT	ACCTGAGGAGGACCAGAG	197	G	ch2	Li et al 2009
b01p03	(TC)18	CACGACGTTGTAAAACGACAAGTACTACTGCACTCGGGA	CACATGCATGATAAAAGCC	153	G	ch3	Li et al 2009
b02c06	(TC)20	CACGACGTTGTAAAACGACCAGTTGCTGCTAAGGATTCT	CCTCGCTATGAGTGGTCTAC	176	G	ch9	Li et al 2009
d02b12	(TAA)6	CACGACGTTGTAAAACGACCAGTCCATCTTCACCTTGAT	TGATCGAGTTACCAAATTTACA	176	G	ch1	Li et al 2009
GB-ZJM-007	(GHC)5 & (GTGC)3	CACGACGTTGTAAAACGACGCCCTGCTTCGTCTTCTT	AAACAACCCCTACCACCG	87-213			Ma et al 2007
ZA01C06	(TG)12	CACGACGTTGTAAAACGACTTGCCGTAGTATATTGGTATTG	GCATTATATGACGAGAAATGG	115	4	0.62	Cai et al 2005
ZA01C15	(CA)33	CACGACGTTGTAAAACGACAAACCAATTAAGTCATTTGCA	CAATGAATAGAAGTCTGTCGG	241	6	0.74	Cai et al 2005
ZA01M20	(GA)7	CACGACGTTGTAAAACGACCCATGTTCACTTTCCTCATT	GAATGGATTGGGTCTAAACA	119	3	0.46	Cai et al 2005

G Polymorphism
S Monomorphism
M Multi-copy
N No amplification