

Genetic Divergence of Bermudagrass (*Cynodon spp.*) Population Using ISSR Markers

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Abstract: Bermudagrass is a widely distributed turf grasses use in Malaysia. Nevertheless, the genetic variation on molecular level of bermudagrass was insufficient. In this study, a sum of 15 cultivars were collected from different accessions in Malaysia and being analyzed using Inter-simple Sequence Repeat Markers (ISSR). A total of 172 fragments were generated from 15 selected ISSR primers. There are 164 polymorphic bands with 95.3% of polymorphism. Fragment size ranged from 120 to 1867bp. The cultivars were clustered into 5 major groups at coefficient level of 0.5 through arithmetic average (UPGMA) cluster analysis of Jaccard's similarity coefficient. The genetic similarity coefficient was range from 0.25 to 0.61 with an average of 0.43. Satiri and Melaka cultivars were clustered separately from their own hybrid and common clusters, respectively. These findings indicated that ISSR marker is an effective molecular method use to study genetic variation of bermudagrass and could be used for varietal development program.

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1. Introduction

Turfgrasses are among the most important industries in many countries including Malaysia because of the development in landscaping and recreation amenity (Juraimi, 2001). Turf grass, as an important element to the landscape, serves the functions as beautification and its attractiveness is suitable for mental health, more specifically; the aesthetic effect of parks, gardens and lawns. Turfgrasses, especially sport turf, play an important role by providing cushioning effect that could help reduce injuries to participants and improve playability.

Bermudagrass, *Cynodon* species are the major turf grasses used for sport fields, lawn, park and other more purpose. It has also moderate tolerance to shade, drought salinity tolerance (Uddin et al, 2011, 2012). For our information, bermudagrass possess high quality for giving superior morphological characteristic, especially for hybrid bermudagrasses (Pannkuk, 2011; Brosnan, 2008). It has a basic chromosome number of X=9, and the reported predominant cytotypes ranging from diploid ($2n=2x=18$), tetraploid ($2n=4x=36$), triploid of hybridization ($2n=3x=27$) and recently discovered of pentaploid ($2n=5x=45$) and hexaploid ($2n=6x=54$) cultivars (De Silva and Snaydon, 2011; Wu et al., 2006; Kang et al., 2008).

In present-day, PCR-based DNA markers such as Inter Simple Sequence Repeat (ISSR), Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), Sequence-related Amplified Polymorphism (SRAP) and Random Amplified Polymorphic DNA (RAPD) technique had been carried out to determine the genetic diversity of bermudagrass (Tan et al., 2010, Wu et al., 2004, Huang et al., 2010, Wang et al., 2011, Etemadi et al., 2006). The use of molecular marker can help in characterizing and managing genetic diversity from classic morphological characterizing method (Sergio and Gianni, 2005; Cortese et al., 2010). It is also more reliable to do the analysis of genetic variation instead base on phenotype only because environment can influence gene expression (Henry, 1997; Rahman et al., 2011).

ISSR is one of the simple, quick and cost effective methods of PCR based techniques which combine most of the advantage from SSR, AFLP and RAPD (Pradeep Reddy et al., 2002; Wang, 2002, Sucher et al., 2012). ISSR amplifies DNA segment present in between two opposite direction of identical microsatellite repeat region (Pradeep Reddy et al., 2002). It ad been successfully identified wide variety of cultivars such as lotus, mango and sweet potato (Chen et al., 2008; Luo et al., 2011; Li et al., 2008).

The identification of each bermudagrass cultivar is important for turf grass selection in breeding programs and field establishment (Pannkuk, 2011). There are many previous research had done on bermudagrass genetic diversity in different country such as China, Korea and Australia (Kang et al., 2008; Li et al., 2010; Jewell et al., 2012). However, the information and study on genetic diversity traits of bermudagrass in Malaysia was limited. Molecular marker-assisted analysis can help breeders to improve turf grass cultivar characteristic, planting stock certification and off-type identification (McCullough, 2004), which provides more accurate data than the traditional morphological classification. Thus, the objective of this study is to elucidate the genetic diversity of bermudagrass using ISSR markers.

2. Material and Methods

Plant materials

Fifteen bermudagrass cultivars from different accession in Malaysia were used in this study. All of the Bermudagrass were the collection of Universiti Putra Malaysia (UPM) which planted in Unit Latihan Turf, UPM. There are eleven hybrid bermudagrasses, namely Satiri (S), C4, C5, C6, C8, C9, C11, C12, C13, C14, C15 and four common bermudagrasses, namely Melaka (M), Bermuda419 (B419), Greenless Park (GP) and Ladang10 (L10). Young bermudagrass leaves were collected early in the morning before 8am in Unit Latihan Turf, UPM. The collected leaves were stored in the freezer immediately at -20°C until DNA extraction.

DNA Extraction

DNA was extracted using the CTAB method (Doyle, 1991) with some modification. 0.1 g of Bermuda grass leaves was ground in liquid nitrogen and taken into 2ml microcentrifuge tube. 800ul of extraction buffer [100mM Tris-HCl pH8.0, 20mM EDTA pH8.0, 1.4M NaCl, 2% (w/v) CTAB, 2% (w/v) PVP (polyvinylpyrrolidone) and B-mercaptoethanol] was added and mixed gently. It is incubated at 65°C for 1 hour. The sample was added with 600ul chloroform: isoamyl alcohol (24:1 (v/v)) and were centrifuged at 13,000rpm for 5 minutes. The supernatant were transferred to a new microcentrifuge tube and 600ul of cold isopropanol was added into supernatant. After 30 minutes of incubation in freezer, the genome DNA in tube was precipitated by centrifugation at 13,000rpm for 10 minutes. The resulted pellet was washed with 70% ethanol, air-dried and re-suspended in 50ul TE buffer [10mM Tris-HCl pH8.0, 1mM EDTApH8.0] and 1ul RNase. The quality of DNA sample was evaluated by spectrophotometer reading of the absorbance at 260 and 280nm. DNA samples were stored at -20°C prior to ISSR analysis.

DNA quantification

DNA was quantified by using nano-drop spectrophotometer (ND2000C, spectrophotometer). For re-quantification, DNA was run on 1% agarose gel electrophoresis using 1x TBE buffer at 80 voltage in 30 min and visualized under UV light by staining with 1 µl Midori Green DNA stain. DNA was diluted with TE buffer to a concentration of 60 ng/µl for PCR analysis and kept in a refrigerator of -20°C.

ISSR-PCR protocol

A total of 24 ISSR primers were screened for the amplification analysis. The Polymerase Chain (PCR) was performed in a final volume of 15ul containing 14ul of Dream Taq™ Green PCR Master Mix (2X) Fermentas (Thermo Fisher Scientific Inc.) and 1ul genomic DNA. All reactions were carried out on a BIO-RAD T100™ Thermal Cycler using the following profile: 3 min at 95°C, 35 cycles of 30s at 95°C, 1min at annealing temperature 56°C, 2 min at 72°Cs and final step 10 min at 72°C.

Data analysis

Visible polymorphic banding of ISSR markers were chosen for analysis. Present and absence of bands were scored as 1 and 0, respectively. The data was recorded in Microsoft Excel Spreadsheet and polymorphisms of ISSR markers were calculated. Scoring of 15 selected ISSR markers were then analyzed using Numerical Taxonomy and Multivariate Analysis System (NTSYS) program version 2.1 (Rohlf, 2002). Similarity matrix was generated using Jaccard's co-efficient of similarity, while dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). The binary data were preceded to Principle Component Analysis (PCA).

3. Results

ISSR analysis

Genetic diversity of fifteen collected bermudagrass was analyzed using Inter Simple Sequence Repeat (ISSR) DNA markers. A total of 25 ISSR primers were screened for their ability to amplify the genomic DNA in collected bermudagrass. There are 15 selected ISSR primers generated a total of 164 polymorphic bands with 95.35% of polymorphism (Table 1). UBC808 generated a minimum of 4 bands and the maximum 17 bands by UBC815 and ISSR22. Nine primers of UBC808, ISSR9, ISSR10, UBC873, UBC880, ISSR20, ISSR21, ISSR22 and ISSR 25 produced 100% polymorphic bands. The ISSR amplification profile produced by UBC807, ISSR22, ISSR25 and ISSR10 are shown in Figure 1.

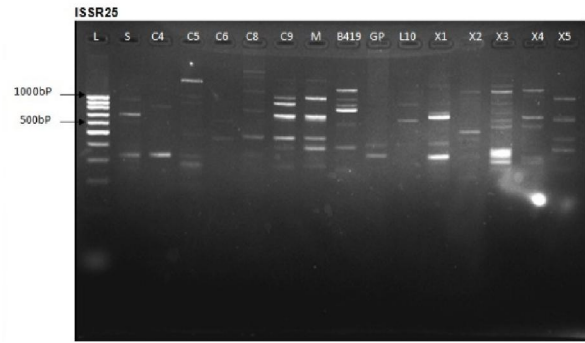
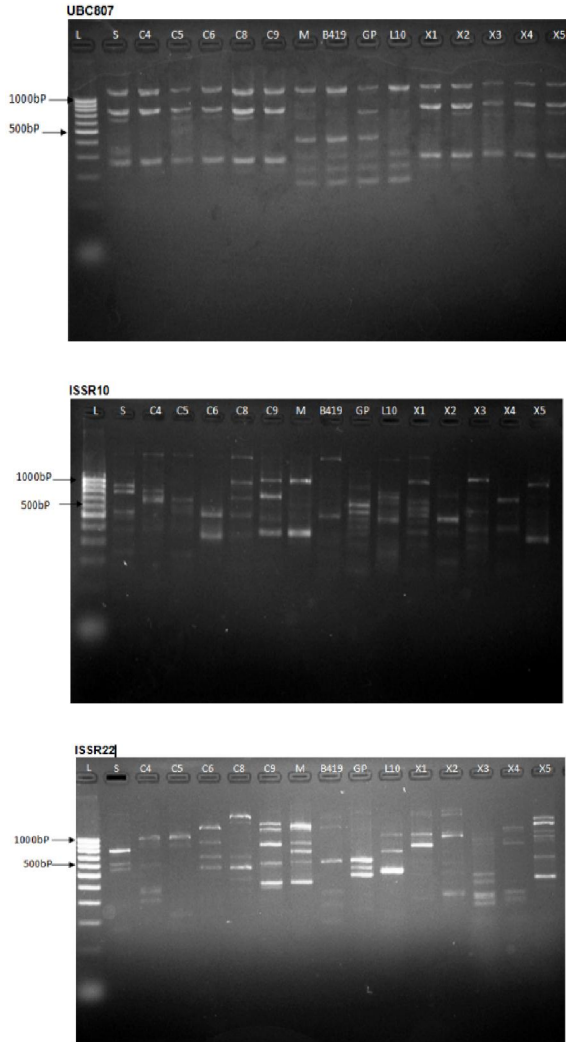


Figure 1. Banding pattern of 15 Bermudagrass using UBC807, ISSR10, ISSR22 and ISSR25

Cluster Analysis

Jaccard's Genetic Co-efficient shows the high level of genetic variation among 15 Bermudagrass cultivars which range between 0.25 and 0.61 (Table 2). The highest genetic similarity coefficient 0.61 was observed between C6 and C8, while the lowest genetic similarity coefficient 0.25 was between C4 and Melaka cultivar. It has an average of 0.43 genetic similarities. A dendrogram was generated (Figure 2) which signifies the overall genetic relationship among the bermudagrass cultivars. According to the analysis, 15 cultivars of bermudagrass were grouped in five main clusters at coefficient level of 0.5. Cluster I consisted of Satiri. Cluster II consisted of C4, C6, C8, X2, C9 and X5. Cluster III consisted of C5, X1, X3 and X4. Cluster IV consisted of Melaka cultivar. Lastly, Cluster V consisted of Bermuda419, Ladang 10 and Greenless Park.

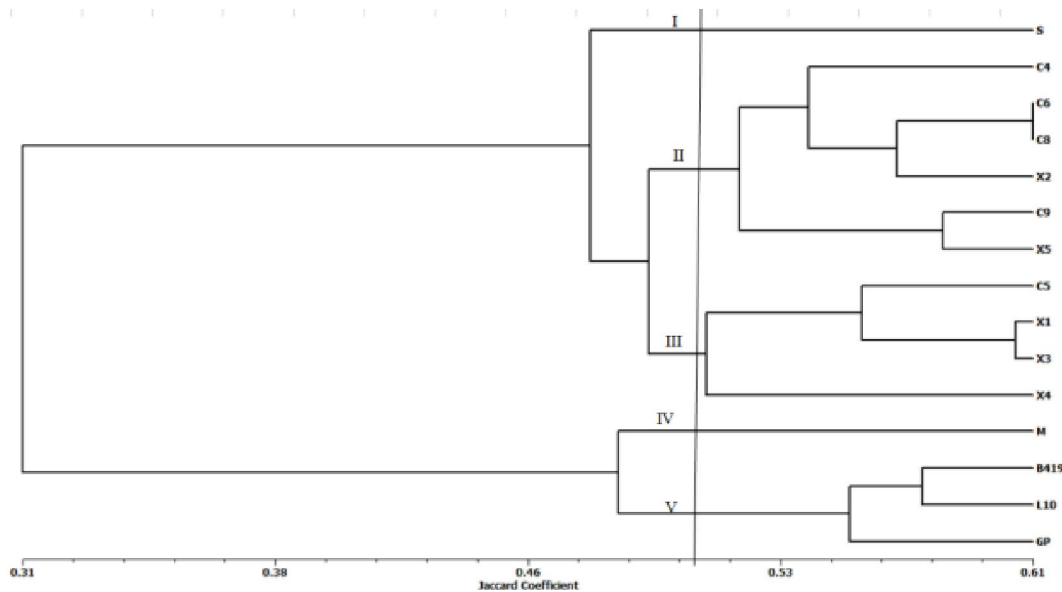


Figure 2. Dendrogram of genetic diversity for 15 different Bermudagrass from cluster analysis

Principle Component Analysis (PCA)

In three-dimensional graph of PCA, 15 cultivars of bermudagrass were also cluster into five groups (Figure 3). It shows a distinct difference between common and hybrid bermudagrass cultivars. All common bermudagrasses are in the same cluster except Melaka cultivar. Hybrid bermudagrass cultivars were divided into three clusters, in which 'Satiri' group as one cluster. In PCA, the first three principle component (PC) indicated 63% of the total variation among fifteen bermudagrass cultivars. The values of the first three PC were 46.91%, 10.98% and 5.38% variation.

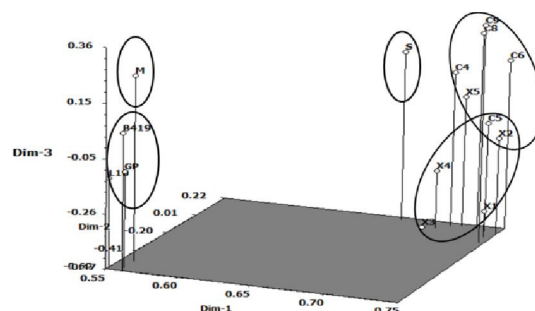


Figure 3. Three dimensional graph showing genetic similarity coefficient among 15 Bermudagrass

Table 1. List of primers used for ISSR analysis, number and percentage of polymorphism

| | Nucleotide Sequence (5'-3') | Length of Amplified Bands | Annealing Temperature (°C) | Number of Amplified Bands | Number of Polymorphic Bands | Percentage of Polymorphism |
|--------------|-----------------------------|---------------------------|----------------------------|---------------------------|-----------------------------|----------------------------|
| UBC808 | AGAGAGAGAGAGAGAGC | 363-900 | 58.9 | 4 | 4 | 100 |
| UBC807 | AGAGAGAGAGAGAGAGT | 181-1267 | 57.2 | 10 | 9 | 90 |
| UBC811 | GAGAGAGAGAGAGAGAC | 240-1400 | 56.6 | 10 | 9 | 90 |
| UBC815 | CTCTCTCTCTCTCTG | 223-1500 | 56.7 | 17 | 15 | 88.24 |
| ISSR9 | AGAGAGAGAGAGAGAGGT | 209-1360 | 60.0 | 13 | 13 | 100 |
| ISSR10 | ACACACACACACACCG | 122-1500 | 65.1 | 13 | 13 | 100 |
| UBC809 | AGAGAGAGAGAGAGGG | 120-929 | 58.3 | 10 | 8 | 80 |
| UBC864 | ATGATGATGATGATGATG | 283-948 | 52.9 | 7 | 6 | 85.71 |
| UBC873 | GACAGACAGACAGACA | 145-1500 | 57.8 | 13 | 13 | 100 |
| UBC880 | GGAGAGGAGAGGAGA | 373-1600 | 59.6 | 10 | 10 | 100 |
| ISSR20 | CACACACACACACATA | 275-1460 | 59.0 | 8 | 8 | 100 |
| ISSR21 | CACACACACACACATC | 273-1460 | 63.5 | 13 | 13 | 100 |
| ISSR22 | ACACACACACACACGA | 236-1867 | 63.8 | 17 | 17 | 100 |
| ISSR24 | AGAGAGAGAGAGAGAGGC | 245-1329 | 61.6 | 13 | 12 | 92.31 |
| ISSR25 | ACACACACACACACGT | 275-1675 | 64.1 | 14 | 14 | 100 |
| Total | | | 172 | | 164 | 95.35 |

Table 2. Jaccard's similarity matrix of 15 different Bermudagrass

| | S | C4 | C5 | C6 | C8 | C9 | M | B419 | GP | L10 | X1 | X2 | X3 | X4 |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| C4 | 0.49 | 1.00 | | | | | | | | | | | | |
| C5 | 0.55 | 0.49 | 1.00 | | | | | | | | | | | |
| C6 | 0.55 | 0.55 | 0.56 | 1.00 | | | | | | | | | | |
| C8 | 0.48 | 0.52 | 0.50 | 0.61 | 1.00 | | | | | | | | | |
| C9 | 0.47 | 0.52 | 0.52 | 0.54 | 0.59 | 1.00 | | | | | | | | |
| M | 0.27 | 0.25 | 0.35 | 0.31 | 0.31 | 0.47 | 1.00 | | | | | | | |
| B419 | 0.26 | 0.34 | 0.29 | 0.29 | 0.30 | 0.37 | 0.54 | 1.00 | | | | | | |
| GP | 0.28 | 0.29 | 0.30 | 0.29 | 0.31 | 0.32 | 0.48 | 0.53 | 1.00 | | | | | |
| L10 | 0.28 | 0.29 | 0.32 | 0.29 | 0.27 | 0.32 | 0.43 | 0.57 | 0.57 | 1.00 | | | | |
| X1 | 0.45 | 0.48 | 0.58 | 0.52 | 0.47 | 0.47 | 0.32 | 0.31 | 0.36 | 0.37 | 1.00 | | | |
| X2 | 0.48 | 0.56 | 0.47 | 0.56 | 0.58 | 0.49 | 0.30 | 0.34 | 0.34 | 0.30 | 0.59 | 1.00 | | |
| X3 | 0.40 | 0.43 | 0.53 | 0.46 | 0.44 | 0.43 | 0.28 | 0.31 | 0.32 | 0.29 | 0.60 | 0.51 | 1.00 | |
| X4 | 0.43 | 0.48 | 0.48 | 0.50 | 0.46 | 0.46 | 0.31 | 0.30 | 0.29 | 0.30 | 0.51 | 0.54 | 0.54 | 1.00 |
| X5 | 0.46 | 0.51 | 0.52 | 0.51 | 0.53 | 0.58 | 0.32 | 0.25 | 0.30 | 0.29 | 0.49 | 0.48 | 0.51 | 0.53 |

4. Discussions

Genetic variation among bermudagrass cultivars was able to detect using ISSR markers. The choices for selecting efficient primers are important. There is high level of genetic diversity in bermudagrass with 95.35% of polymorphism. Number of

polymorphic bands observed from 15 selected ISSR markers was range from 4 to 17, with an average of 10.93. Using ISSR markers as genetic tool to detect bermudagrass polymorphism was also successful in different papers by Li et al. (2010) and Farsani et al. (2011).

Similarity matrix of Jaccard's coefficient was varies among different cultivars. The highest genetic similarity coefficient was among C6 and C8 hybrid cultivar. This means that C6 and C8 have the highest similarity in morphological appearance as there is significant correlation between morphological and molecular diversity (Rahman et al., 2011). The lowest genetic similarity coefficient of C4 and Melaka cultivar indicate a distance genetic similarity between a hybrid and common cultivars of bermudagrass.

On the other hand, UPGMA cluster analysis on Jaccard's genetic similarity coefficient showed genetic variation in different clusters through illustration of dendrogram. According to dendrogram constructed using ISSR markers, there are 5 clusters in total at 0.5 similarity coefficient level, in which 3 clusters belong to hybrid triploid bermudagrass and 2 clusters for common tetraploid bermudagrass (*Cynodon dactylon*). Hybrid 'Satiri' and Melaka cultivar each appears as an independent cluster in the analysis. This phenomenon of clustering indicate that Satiri and Melaka cultivar have a slight genetic divergent different from their own hybrid and common clusters, respectively. The three dimensional graph of Principle Component Analysis (PCA) also clearly shown the genetic variation among each cultivars, supporting data from cluster analysis. Similarly, the grouping pattern of clustering was shown corresponds well with the PCA in others papers by Huang et al. (2010); Li et al. (2010) and Senthil Kumar et al. (2009).

Hybrid 'Satiri' was a product from a long term breeding program of Universiti Putra Malaysia. It is launched and commercialized in 2008. In this study, it shows a slight genetic different and unique in DNA finger prints from the other two clusters of hybrid bermudagrass. Thus, this indicated that hybridization and selection do enhance the genetic diversity as stated by Rauf et al. (2010). Besides Melaka cultivar, common bermudagrass namely, Bermuda 419, Ladang 10 and Greenless park cultivars which were collected almost in the same geographical region grouped in a same cluster. This result has indicated an agreement to the statement by Li et al. (2010) and Farsani et al. (2011) in which the accession in the same location and similar environment generally were clustered into the same subgroup.

From this research, it is clearly shown that hybridization and geographical region different can affect genetic diversity of a species. Gulsen et al. (2009) reported that different geographical distribution can effect genetic variation in bermudagrass. In some cases, genetic variation may due to off-types somatic mutation which usually occurs in golf courses greens and fairways of 'Tifgreen' and 'Tifdwarf' bermudagrasses (Busey, 1997). Mutation is a genetic shift in genome and new alleles were produced (Rohlf,

2002). Variation in these genetic traits can applied as a source in breeding program for better improvement quality of Bermudagrass.

5. Conclusions:

The genetic relationship of 15 bermudagrass collected from different accession in Malaysia was analyzed using 172 fragments generated by 15 ISSR primers. The genetic similarity of Bermudagrass was range from 0.25 to 0.61 from Jaccard's similarity matrix, with 95.35% of polymorphism. UPGMA dendrogram shows 5 major clusters of bermudagrass. The result indicated that ISSR marker is an effective molecular method use to study genetic variation of bermudagrass in Malaysia. On the other hand, genetic variation in bermudagrass was proved due to hybridization and geographical difference. This study provides useful information for future uses and genetic conservation documentation purposes.

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