Genetic Diversity of Carpetgrass Germplasm Based on Simple Sequence Repeat Markers

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Abstract. Carpetgrass [*Axonopus compressus* (Sw.) Beauv.] is an important warm-season perennial turfgrass that is widely used in tropical and subtropical areas. The genetic diversity of 63 carpetgrass accessions in China was studied using simple sequence repeat (SSR) markers. Fourteen SSR primer combinations generated a total of 49 distinct bands, 48 (97.96%) of which were polymorphic. The number of observed alleles ranged from 2 to 6, with an average of 3.5. Coefficients of genetic similarity among the accessions ranged from 0.24 to 0.98. Unweighted pair-group method with arithmetic means (UPGMA) clustered the 63 accessions into three groups, and not all samples from the same region belonged to the same group. SSR markers will promote marker-assisted breeding and the assessment of genetic diversity in wild germplasm resources of carpetgrass.

Carpetgrass [*Axonopus compressus* (Sw.) Beauv.] is a perennial warm-season turfgrass with many excellent characteristics, such as easy establishment and low management. Because of these traits, carpetgrass is often used to conserve soils and highway slopes and is planted as turf in common areas, sports fields, and shady areas in south China and around the world (Xi et al., 2004a). All individuals of carpetgrass are polyploid, with chromosome numbers $2n = 4x = 40$, $2n = 5x = 50$, and $2n = 6x = 60$ (Delay et al., 1950), and the species is commonly cross-pollinated because of a self-incompatibility mechanism (Hanna and Burton, 1978). Carpetgrass is widely distributed in tropical and subtropical regions (27°N–27°S) and adapted to a wide range of soil pH (pH 4.1–7.1) and soil types (Heath et al., 1985; Liu, 2010). Natural hybrids have provided a very genetically rich germplasm of wild carpetgrass. Previous study revealed that carpetgrass accessions of south China had unique morphological and agronomic traits (Liao et al., 2011). However, the genetic diversity of wild carpetgrass germplasm

resources has been rarely explored, especially at the molecular level (Xi et al., 2004a). Thus, the purpose of this study was to assess this variation using molecular markers to advance the genetic breeding of carpetgrass.

Most studies of carpetgrass have focused on its genetic characteristics and resistance (Samarakoon et al., 1990; Smith and Whiteman, 1983; Uddin et al., 2009; Xi et al., 2006), but there is little information regarding its diversity on which to form a complete set of evaluation criteria. The existing studies are limited in the breadth of accessions examined (Xi et al., 2004a). The development of molecular biology has provided popular techniques to assess plant genetic diversity and analyze genetic variation using molecular markers. For lawn grass, analyses by both domestic and foreign scholars have identified a variety of molecular markers that can be applied to these tasks (Wang et al., 2009; Weng et al., 2007; Wu et al., 2006).

To explore genetic characteristics and diversity in plants, many types of molecular markers are available, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP), and SSR (SSRs; also known as microsatellites) (Schlotterer, 2004; Weising et al., 2005). RAPD, AFLP, and SRAP are dominant markers that cannot detect whether an allele is heterozygous or homologous. Although RFLPs are codominant, the procedure is

complex and requires high-quality DNA, so it is not conducive to automation for a large number of samples (Song et al., 2014). Therefore, SSRs have become the genetic markers of choice for many plant species because of their advantages over RFLPs, RAPDs, AFLPs (Pejic et al., 1998), and other DNA markers, such as intersimple sequence repeats (Wang et al., 2013). The advantages include uniform genome coverage, high polymorphism rates and informativeness, codominance, and the availability of specific polymerized chain reaction (PCR)-based assays. SSRs have been useful for other warm-season turfgrasses (Harris-Shultz et al., 2013; Madesis et al., 2014).

Axonopus has not been previously studied using microsatellite markers, therefore, we examined the genetic diversity of carpetgrass germplasms using SSRs. The results will provide important methods and technologies for future research. The objectives of this study were 1) to use an SSR marker system to study the genetic relationships among 63 carpetgrass accessions from China and 2) to describe the genetic variation in the accessions.

Materials and Methods

Plant material. Sixty-three carpetgrass accessions were collected from Hainan, Guangdong, Guangxi, Yunnan, Guizhou, and Fujian provinces of China (Table 1). The materials were grown under field conditions at Hainan University, Danzhou, Hainan Province, China (19°31'N, 109°34'E, altitude 131 m). The area has a tropical monsoon climate with an average annual rainfall of 1,815 mm. The annual average sunlight exceeds 2,000 h with a solar radiation intensity of 34,244.6 Lux (14,012.2–53,587.4 Lux). The field temperature ranged from 9.0 °C to 38.6 °C (average, 24.0 °C). All accessions were identified as carpetgrass based on the morphological characteristics described by Liu (2010).

Vegetatively propagated carpetgrass accessions (cultivars) were obtained from the suppliers listed in Table 1. All wild accessions were collected by gathering healthy stolons from the field. For each accession, ≈40 healthy stem segments with two internodes each were planted in a regular array in a plot of 0.5 × 0.5 m². Plots of different accessions were separated by 50 cm, and each plot was trimmed to prevent plot-to-plot contamination. The plots were frequently mowed to a height of 50 mm to ensure healthy turf and a supply of newly expanded leaf blades.

Although carpetgrass is widely distributed in south China, studies on the asexual reproduction characteristics of widespread indigenous accessions are lacking. The purpose of this study was to assess the genetic variation and the asexual reproduction characteristics among 63 carpetgrass accessions from China, the following measurements were preliminarily assessed: vertical growth (was calculated as the height of the grass layer divided by 28 d), secondary stolon branches per unit perimeter [was calculated as the number of secondary branches in the

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plot divided by the plot perimeter (2.0 m), the same as below], horizontal growth rate of a single branch (was calculated as the total length of stolons in the plot divided by 28 d),

coefficient of expansion, and numbers of both primary.

DNA extraction. Genomic DNA of each carpetgrass accession was extracted from

fresh, healthy leaf samples following the CTAB (Hexadecyl trimethyl ammonium Bromide) method described by Doyle and Doyle (1987). DNA quality was checked in 1.5% agarose gels, and DNA concentrations were determined by reference to a sample of known concentration. Genomic DNA was diluted to a final concentration of 50 ng·μL⁻¹ and stored at -20 °C for further analysis.

SSR primers. We used Roche 454 sequencing combined with the magnetic bead enrichment method of FIASCO to isolate SSR markers from the carpetgrass genome. A total of 1,942 microsatellite loci were identified in 53,193 raw sequencing reads. To test the primer amplification efficiency, we randomly selected 100 microsatellite sequences to design primer pairs and deposited these sequences in GenBank (KM110853–KM110930) (Table 2). Fourteen of the 100 primer pairs were selected to amplify polymorphisms in the 63 carpetgrass accessions from China (Table 1).

Primer screening and DNA amplification. Fourteen primer pairs with clear gel bands, reproducible fragments, and strong signal were used for further analyses. PCR amplification followed previously established protocols (Wang et al., 2007). A 20-μL volume of PCR mixture consisted of 3 μL of 10x buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂), 1.5 μL dNTPs, 1.0 μL each forward and reverse primers, 0.2 μL *Taq* DNA polymerase (TaKaRa), 12.3 μL ddH₂O, and 1.0 μL DNA template (50 ng·μL⁻¹). The PCR amplifications were carried out in a TECHNE TC-412 machine (Bibby Scientific, Staffordshire, UK) with the following protocol: 94 °C for 5 min for initial denaturation; 37 cycles of

Table 1. Sixty-three carpetgrass accessions used in the study.

No.	Accession No.	Locations	No.	Accession No.	Locations
1	A2 (Huanan)	Chinese Academy of Tropical Agricultural Sciences	33	A83	Xiamen, Fujian
2	A3	Dingan, Hainan	34	A84	Zhangpu, Fujian
3	A4	Dingan, Hainan	35	A86	Changtai, Fujian
4	A5	Danzhou, Hainan	36	A87	Daxin, Guangxi
5	A7	Baisha, Hainan	37	A88	Fusui, Guangxi
6	A15	Qionghai, Hainan	38	A94	Chongzuo, Guangxi
7	A16	Sanya, Hainan	39	A95	Gelinnada
8	A19	Wenchang, Hainan	40	A97	Tengchong, Yunnan
9	A23	Wanning, Hainan	41	A98	Huaiji, Guangdong
10	A25	Guigang, Guangxi	42	A99	Huaiji, Guangdong
11	A28	Chengmai, Hainan	43	A100	Fogang, Guangdong
12	A30	Qiongzong, Hainan	44	A101	Guiping, Guangxi
13	A34	Ledong, Hainan	45	A102	Tunchang, Hainan
14	A37	Danzhou, Hainan	46	A103	Wuzhishan, Hainan
15	A38	Haikou, Hainan	47	A105	Changjiang, Hainan
16	A41	Menghai, Yunnan	48	A106	Hepu, Guangxi
17	A42	Ledong, Hainan	49	A107	Hepu, Guangxi
18	A45	Yingde, Guangdong	50	A108	Hepu, Guangxi
19	A47	Hekou, Yunnan	51	A111	Yulin, Guangxi
20	A49	Ceheng, Guizhou	52	A113	Leizhou, Guangzhou
21	A51	Zhangpu, Fujian	53	A114	Haikou, Hainan
22	A52	Wuzhou, Guangxi	54	A116	Maoming, Guangdong
23	A54	Huizhou, Guangdong	55	A118	Dianbai, Guangdong
24	A57	Zhangzhou, Fujian	56	A120	Yulin, Guangxi
25	A58	Guangzhou, Guangdong	57	A121	Leizhou, Guangzhou
26	A59	Nanjing, Fujian	58	A122	Xuwen, Guangdong
27	A63	Mangshi, Yunnan	59	A123	Lianjiang, Guangdong
28	A67	Mangshi, Yunnan	60	A126	Suixi, Guangdong
29	A70	Ruili, Yunnan	61	A139	Qiongzong, Hainan
30	A72	Ruili, Yunnan	62	A140	Shangsi, Guangxi
31	A81	Changtai, Fujian	63	A141	Wuzhishan, Hainan
32	A82	Fusui, Guangxi			

Table 2. Amplification information of simple sequence repeat primers in carpetgrass accessions.

Primer	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Allele Size (bp)	No. of alleles	No. of polymorphic bands	GenBank Accession no.
ID19	F: TTATAGGCGCTCACAAAGCG R: CTCCTGCTCCTGCTGCTACT	(TAG)5(GAG)6A(AGG)5	60	184–205	2	1	KM110853
ID21	F: TTGTTCTCAGTGGTCTCGCT R: TAATGCACGCCCATAGAACA	(AC)15(AT)7	48	315–373	2	2	KM110855
ID25	F: AGCATCGTCGAAAAACCTGT R: TTGCATGAAAGTAAAGCAATGAA	(ATG)17	60	231–294	3	3	KM110859
ID34	F: TGCTTGGCCTCTAGCCTACT R: ACCAGCAGATGTGGTTGATT	(AAC)12...(AAC)11(AAT)5(CAT)5	60	198–252	4	4	KM110868
ID41	F: GGTCCATCATCTCCAAGAA R: ACTTGGAGGTCACTTGGCAT	(AAG)5...(TGA)6	60	344–353	3	3	KM110875
ID67	F: TGAAGTCAATTAGGATTTTATGGG R: TGCGAGATGAGTTCGAGTATC	(TATG)29...(TATG)5	60	304–400	3	3	KM110901
ID73	F: TTCCCCACTAAAAATGACGG R: CAATCTTATCCGCCATGAAA	(GT)6(AT)8(GT)13	56	173–189	3	3	KM110907
ID79	F: GCTTTCTCGAGAGTCATCCG R: TGAATGATCAGAATGTGGAGTTCT	(TCT)16	56	137–188	4	4	KM110913
ID84	F: AGGGCACCAGGGTTAAAGAT R: TCATGGAGGTGCCATGAAA	(CA)6...(CA)7	48	275–299	5	5	KM110918
ID85	F: GCCCACGAACCTTTCTCAGT R: TGTTTGCTTGCTCCCTTCTCA	(TCTATC)7	48	244–305	5	5	KM110919
ID87	F: AGGGGGCAGCTCATTTTTAT R: ATTCAGGACTCGGTTGATGC	(AT)6(AC)27	56	290–380	6	6	KM110921
ID91	F: GCTCCACATCTTTCTGCGAT R: ACCATATGTACAAGGTGCTAGTTAGG	(TATG)13	60	253–269	4	4	KM110925
ID94	F: GGCCATATAAGGTGACGCAT R: TTTTCATGGTTGCCAAATCA	(AAT)11(AAC)15	60	329–389	2	2	KM110928
ID96	F: GAGGGGGCTAGGCATTTTAG R: TGAAATGCAAGCACACACAA	(TG)8...(TG)9	56	192–220	3	3	KM110930
Total					49	48	
Average					3.5	3.4	

denaturing at 94 °C for 45 s, annealing at 48.0–60.0 °C for 45 s, and elongation at 72 °C for 78 s; 72 °C for 7 min for a final extension; and holding at 4 °C. The amplified products (3 µL) were separated on an 8.0% nondenaturing polyacrylamide gel (in 1x TBE buffer) at 200 V for 2.5 h at room temperature using a Hoefer vertical-gel apparatus (JY-SCZ7). After electrophoresis, the gels were silver-stained for detection (Wang et al., 2007). Allele sizes were estimated using a 100 base pair (bp) DNA ladder (Takara, Dalian, Liaoning, China) as reference.

Data analysis. Bands of the amplified fragments were recorded as present (1) or absent (0). According to method of Nei and Li (1979), genetic similarity coefficients (GSCs) of the 63 accessions were calculated by using Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) (v. 2.10s) software (Rohlf, 1993). Genetic polymorphism (P-5%), Nei's gene diversity (He), and Shannon's information index (I) were used to calculate Nei's standard genetic distance coefficients (Nei and Li, 1979) and to construct a UPGMA dendrogram within the sequential agglomerative hierarchical nested cluster analysis (SAHN) module of NTSYS (Sneath and Sokal, 1973).

Results

SSR analysis. Fourteen SSR primer pairs (Table 2) were used to examine the genetic similarities among the 63 carpetgrass accessions. Of a total of 49 amplified fragments, 48 (97.96%) were polymorphic, with an average of 3.5 amplified bands per primer pair (Table 2; Fig. 1). The polymorphism was either 100% (ID21, ID25, ID34, ID41, ID67, ID73, ID79, ID84, ID85, ID87, ID91, ID94, and ID96) or 50% (ID19). PCR product sizes ranged from 100 to 400 bp.

Statistical analysis. The coefficients of genetic similarity among the 63 carpetgrass accessions ranged from 0.24 to 0.98. The closest genetic relationship was between samples from Hepu, Guangxi Province (A106 and A108), with a GSC of 0.98. The lowest GSC of 0.24 was between accessions from Xiamen, Fujian Province (A83) and Wuzhishan, Hainan Province (A141). Overall, the accessions were closely related, and the largest GSC values were from the same or similar geographic locations.

Cluster analysis. The UPGMA cluster analysis of SSR results of the 63 carpetgrass accessions resulted in a dendrogram of genetic relationships that agreed with the pedigrees and geographic origins of the plants. There were three main clusters (I, II, and III) (Fig. 2). Cluster I included 38 accessions from Hainan (16 accessions), Fujian (6), Guangdong (3), Guangxi (5), Yunnan (7), and Guizhou (1) provinces. This group was characterized by excellent turf characteristics, including relatively slow vertical growth (was calculated as the height of the grass layer divided by 28 d); a high coefficient of expansion [was calculated as the total length of stolons in the plot divided by

Fig. 1. Simple sequence repeat amplified with primer ID73 in 63 DNA of carpetgrass, Marker is 100-base pair DNA ladder, material number was showed in Table 1.

Fig. 2. UPGMA clustering for 63 carpetgrass accessions based on simple sequence repeat marker.

the perimeter of plot (2.0 m)]; horizontal growth rate of a single branch (was calculated as the total length of stolons in the plot divided by 28 d); and very high numbers of both primary [was calculated as the number of primary branches in the plot divided by the plot perimeter (2.0 m)] and secondary stolon branches per unit perimeter [was calculated as the number of secondary branches in the plot divided by the plot perimeter (2.0 m)]. Two cluster I accessions, from Hainan (A30) and Guangdong (A58) provinces, had better vegetative propagation traits than the Huanan cultivar (control varieties) used in this study. Cluster II contained 22 accessions from Guangdong (8), Guangxi (7), Fujian (1), Hainan (5), and Yunnan (1) provinces. The asexual reproduction characteristics of this group were generally between those of Clusters I and III, these accessions may be useful in some breeding situations. Cluster III comprised three accessions, one each from Hainan, Guangdong, and Guangxi provinces, with opposite characteristics to those in Cluster I. Not all accessions from the same region clustered in the same group.

Discussion

SSRs have become one of the most widely used types of molecular markers in recent years. They have several advantages over many other DNA markers, such as high polymorphism rates, abundance, genetic codominance, and the availability of specific PCR-based

assays (Pejic et al., 1998). For instance, in this study, some accessions from the same region were similar in morphology but could be distinguished by the SSR markers. Thus, SSRs are very useful molecular markers for studying carpetgrasses in large-scale breeding programs (Tang et al., 2007).

At present, genetic diversity analyses using microsatellite markers have been reported in turf and forage grasses, including bermudagrass [*Cynodon dactylon* (L.) Pers.; Wang et al., 2013], zoysiagrass [*Zoysia japonica* Steud.; Li et al., 2009], and centipedegrass [*Eremochloa ophiuroides* (Munro) Hack; Zheng et al., 2013]. However, there have been no similar publications on carpetgrass. This study is the first assessment of molecular genetic diversity in carpetgrass accessions.

We analyzed the genetic relationships of 63 carpetgrass accessions from China using SSR molecular markers. The GSCs ranged from 0.24 to 0.98. This result suggested that the analyzed germplasms possessed a great amount of variation and represented the diversity of the source germplasms (Chen and Du, 2006). Wu et al. (2006) showed that the GSC ranged from 0.65 to 0.99 (average 0.82) among 119 bermudagrass accessions based AFLP markers. Wang et al. (2010) observed that the GSC ranged from 0.807 to 0.98 (average 0.89) among 59 common carpetgrass [*A. fissifolius* (Raddi) Kuhl.] accessions. These studies and our own data indicated that the carpetgrass accessions possessed great genetic diversity. Carpetgrass has

many ploidy levels ($2n = 40, 50$, and 60) (Delay, 1950), which may increase genetic differentiation (Wang et al., 2010). Based on our results, we will next analyze the ploidy levels of these carpetgrass accessions, because ploidy level might be affected by the environment or by the evolutionary histories of the genotypes (Johnson et al., 1998). Wu et al. (2006) and Wang et al. (2010) reported similar results for the relationship between genetic diversity and ploidy level of bermudagrass and common carpetgrass accessions, respectively.

Of a total of 49 amplified fragments, 48 (97.96%) were polymorphic, with an average of 3.5 amplified bands for each primer pair. The 63 carpetgrass accessions clustered into three groups by UPGMA (Fig. 2), which appeared to be associated with geographic origin and genetic factors, a phenomenon seen in other plant germplasms (Blair et al., 1999; Bornet et al., 2002; Godwin, et al., 1997; Li and Ge, 2001; Tani et al., 1998; Wang et al., 2010; Wang et al., 2013). Similar results were obtained in *Lolium perenne* (Li et al., 2013) and *Agropyron* (Zeng et al., 2013). The reasons for these results might be as follows: first, accessions from different geographical areas clustered into the same group because of environmental pressures leading to long-term adaptation, so some traits were shared among different carpetgrass germplasms (Zeng et al., 2013). Second, carpetgrass has strong vegetative reproduction ability, especially famous to its leanness resistance and extensive management (Xi et al., 2004b). Third, carpetgrass is a perennial, outcrossing grass, which may lead to rich genetic variation in accessions from different regions. Finally, the multiple ploidy levels of carpetgrass may also promote this phenomenon (Delay, 1950).

In conclusion, this study was the first to use microsatellite markers in *Axonopus* and revealed great genetic diversity in carpetgrass germplasms. This project will provide important methods and technology for future research. The microsatellite markers developed here for *Axonopus* could be helpful for different objectives related to genetic diversity, including assisted breeding and assessing the diversity of wild germplasm resources. Therefore, the use of SSR markers to test differences among germplasms was more effective in detecting genomic variation and may provide valuable references for cross breeding.

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