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Characterization of Microsatellite Markers and Their Application to Genetic Diversity Analysis of *Brachypodium sylvaticum* var. *breviglume* from Yunnan, China

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ABSTRACT

Southwestern China is abundant in a large species diversity of grasses and *Brachypodium sylvaticum* var. *breviglume* is unquestionably appropriate to serve as an ideal model to investigate the adaptation, evolution and diversification of grasses in this region. In this study, a total of 37 microsatellite markers were screened and genetic variation was estimated for 100 individuals from the five natural populations of the species. Our results showed that seven of them were polymorphic for the five studied populations, while the other thirty were monomorphic. These seven SSR loci exhibited a high level of genetic diversity among populations, *i.e.* allele number varied from 5 to 24, with an average of 13.29; expected heterozygosity (*He*) ranged from 0.439 to 0.561, with an average of 0.488; percentage of polymorphic loci (*PPL*) ranged from 85.71% to 100%, with an average of 97. 14%. The FCA and UPGMA analyses revealed that the five populations were divided into three clusters. Our results indicate that these newly characterized SSR markers are useful for the exploration of genetic diversity and evolutionary history of the *B. sylvaticum* var. *breviglume* populations in Yunnan, China.

Keywords: *Brachypodium sylvaticum* var. *breviglume*; Microsatellites; Characterization; Genetic Diversity

1. Introduction

Grasses (Poaceae), the fourth widely distributed family, provide the bulk of human nutrition through directly or indirectly approaches. They are thus regarded as an essential factor in addressing the strategic goal of food security. So far, grasses are also proposed to become major sources of renewable energy under the global energy crisis [1]. As their central importance to the global economy is concerned, more research needs to be focused on understanding the biology of grasses.

With rapid development of molecular biology and genomics, DNA markers are widely employed to study genetic origins, invasion, intraspecific hybridization and dispersal modes of grasses [2,3]. These molecular tools which have been continuously used in population genetic studies of grass species include isozymes [4], RFLPs [5], RAPDs [6], ISSRs and AFLPs [7]. The microsatellite markers, also called simple sequence repeats (SSRs), have been proven to be suited for a wide-range of genetic characterization [8]. They possess many of the characteristics required for population genetic studies including multi-allelic, co-dominant, high polymorphism and abundance [9]. The uses of microsatellites of *Brachypodium sylvaticum* (Huds.) Beauv. in Northern America, for example, have shown their power to discriminate dispersal modes and bio-control of the recently identified invasive species in Oregon and California, USA [2,3].

Southwestern China is abundant in a large species diversity of grasses and possesses approximately 888 grasses, accounting for over 65% of number of total grass species of China. The origins of grass diversity, their speciation and subsequent dispersal in this area have long attracted evolutionary biologists and plant taxonomists but remain largely unsolved to date. *B. sylvaticum* var. *breviglume*, a perennial self-compatible

diploid bunchgrass, is widely distributed in Southwestern China [10,11]. The species exhibits divergently ecological adaptation, frequently intraspecific hybridization and high levels of phenotypic variation. It is unquestionably appropriate to serve as an ideal model of evolutionary studies due to fitness-related traits which are easy to investigate, e.g., nonrhizomatous growth habit, rapid life time, compact genome and greenhouse suitability [2,3, 12]. In addition, the plant has long been used as forage by local people in Southwestern China for its high protein component of about 8.1% - 11.8% during the flowering stage [10]. A recent pioneer study has investigated population genetic structure of 25 populations from throughout the native range in Europe, North Africa and the Middle East by comparing with 23 invasive US populations [3]. However, little has been known about levels and patterns of genetic diversity and population structure of *B. sylvaticum* var. *breviglume* in Asia. In this study, we aim to identify and characterize new microsatellite markers for the characterization of genetic diversity and population structure of *B. sylvaticum* var. *breviglume* to obtain a better knowledge of adaptation, evolution and diversification of the species in Southwestern China.

2. Material and Methods

2.1. Plant Material

A total of 100 individuals were taken from the five representative populations of *B. sylvaticum* var. *breviglume* in Northwestern Yunnan, China (**Table 1**). Young and fresh leaves were collected from each individual in June-October, 2010 and 2011. Individual plants were sampled at least 5-m apart to prevent collecting duplicate samples from the same genet. The sampled leaves were dried and stored in silica gel until DNA extraction.

2.2. Methods

2.2.1. DNA Extraction

Genomic DNAs were extracted from the dried leaf tissues of different individuals by using a modified Cetyl-Trimethyl Ammonium Bromide (CTAB) method [13]. 100 mg of dried leaves were ground manually within liquid nitrogen. The mixture was incubated at 65˚C for 1 h and shaken gently for several times during the incubation, followed by chloroform cleaning-up. The DNA pellet was washed by the ethanol (76%), air dried and then suspended in 100 μL of TE buffer [10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0]. The DNA was quantified by gel electrophoresis and stored at −20˚C until use.

2.2.2. Primer Screening and PCR Experiments

Of the thirty-seven microsatellite markers, twelve were firstly developed in *B. distachyon* and used in *B. sylvaticum* (Huds.) Beauv. [2,3,14], while the others were retrieved from the database of *B. distachyon* genome [15] (**Table 2**). PCR reactions were carried out in 10-μL with 2 × Taq MasterMix (Transgen; 0.1 U *Taq* Polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$) and 0.5 μL of each primer and 0.5 μL genomic DNA. Amplifications were performed in an MJ Research P-100 thermal cycler. After an initial denaturing step of 95˚C for 5 min, the PCR procedure was: 95˚C for 50 s, 55˚C for 10 s, 72˚C for 1min, cycled 30 - 35 times, with a final extension of 72˚C for 5 min. Primers were initially screened for 24 individuals of *B. sylvaticum* var. *breviglume*, and the obtained fragments were visualized on 2% agarose. The microsatellites which

Table 1. Ecological and geographical parameters and levels of genetic variation of the five studied populations of *B. sylvaticum* var. *breviglume* across the seven SSR loci.

Population codes	Location	Latitude (N)	Longitude (E)	*N*	*Na*	*Ne*	*PPL*	*Ho*	*He*
TH	Tanhua Mountain, Dayao County, Yunnan Province	25˚57'	101˚13'	20	2.86	2.03	85.71	0.143	0.439
YY	Yangyu Mountain, Eryuan County, Yunnan Province	26˚10'	100˚18'	20	3.43	2.53	100	0.220	0.520
ML	Meili Mountain, Deqin County, Yunnan Province	28˚24'	98˚45'	20	3.29	2.37	100	0.090	0.561
JZ	Jizu Mountain, Binchuang County, Yunnan Province	25˚57'	100˚23'	20	3.29	2.40	100	0.044	0.441
YL	Yulong Mountain, Lijian City, Yunnan Province	27˚00'	100˚11'	20	2.86	2.16	100	0.029	0.481
Average				20	3.14	2.30	97.14	0.105	0.488

N, sample size; *Na*, mean number of alleles; *Ne*, mean number of effective alleles; *PPL*, percentage of polymorphic loci; *Ho*, observed heterozygosity; *He*, expected heterozygosity.

Table 2. Primer sequences and genetic characteristics of 37 microsatellite loci successfully amplified in *B. sylvaticum* var. *breviglume*.

Locus	Primer Sequence (5'-3')	Repeat motif	N_A	Size range (bp)	*Ta* (˚C)	Accession No.
2-3A1	F:AGAGGGATTGCATTGTCATCAG R:TTCGGAGGATAGCTTGGTCACTC	$(GAA)_{21}$	17	236 - 311	55	EF450746
2-6E6	F:TATGAACCACAAGCCCAGAG R:TCCATGTGCCTGAATCTTGA	$(CAA)_{14}$	14	189 - 249	55	EF450757
2-6E8	F:CTGCTTCCTTGCCCACTAAC R:ATTTATGCCGTGTGGGAGAA	$(GA)_{18}$	24	181 - 283	55	EF450765
3-2E3	F:TTGCGAGGCACGTATGTCTA R:ATCTTGTGCTTCATGGCAGA	$(GGTT)_3$	7	155 - 187	55	EF450751
3-2B2	F:GACAACTCTACTGTGCATGAATTTG R:AGGCTTGGAGCTCATACCAG	$(GTTT)_5$	16	103 - 179	55	EF450754
BS537	F:AGCTTCACCCAGCTGCTCAT R:GTGAGTCGAATAAGCCGGAA	$(CAG)_5$	10	139 - 190	55	JQ307463
BS545	F:CCAGCATCAAGCCAACAATAATC R:TCGTGGATGACGAGCTG	$(CAC)_5$	5	156 - 168	55	JQ307465
BS2	F:CACCCCCTTTCCATTTACCT R:AACCGAAGTTGCCGACTCTA	$(TC)_7$	1	274	55	JQ307441
BS3	F:CCTCGGGTGATCGATTCTTA R:CGTTCAGCTCCTCGTAGTCC	$(GGC)_6$	1	117	55	JQ307442
BS14	F:ACTTGGAACGAAAAGAGGGG R:AGTTGATGGACTGGAGCTGG	$(CGC)_6$	1	280	55	JQ307443
BS18	F:GAAAGCAACACGCCTCTACC R:CGCTACTATGGGTTTCCTGC	$(GACGCA)_5$	1	132	55	JQ307444
BS21	F:AAGAGGGCCATATCTCCAGG R:CATACAGTGGTATCAGCGCC	$(GGC)_7$	1	155	55	JQ307451
BS23	F:AAGATGAAGCTTGCGCTCTC R:CACTGCTGGACGAGGTAGAA	$(TCC)_5$	1	113	55	JQ307445
BS46	F:CAAGCTTAGGGTCCTCCTCC R:TAGAGATGGATCGGAATCGG	$(TAGC)_6$	1	143	55	JQ307446
BS80	F:CGAACAAACCCTTGGTCAAA R:CAGGGCGCTTACAGGATAGA	$(TC)_8$	1	105	55	JQ307447
BS95	F:GTCCTCCATCTCGAAGGCTC R:ATCAGAACAAACTGACCGGC	$(CTT)_3$	1	590	55	JQ307450
BS142	F:AGCAAGCAAAGCAACTAGGC R:AGTTCGATCTACCGTCCAGG	$(CTTT)_3$	1	428	55	JQ307452
BS207	F:TTCTCCTTACGCGGATCTGT R:GAATCCGGATCTCGATCTCA	$(TTC)_4$	1	380	55	JQ307453

Continued

BS265	F:AACCCAAACCCAAACCCTAC R:AGGGTGGACTCCTTCTGGAT	$(CTT)_4$	1	420	55	JQ307454
BS286	F:CAGTTCAAAGACTCTCCGGC R:GATGGACTTAAGCGAGCAGG	$(TA)_7$	1	206	55	JQ307455
BS341	F:CTCCGCTGTCTCTCCACTCT R:ATACCGTCGAGCACCTCAAG	$(GCC)_6$	1	200	55	JQ307448
BS368	F:GACGAGCACGAAAACTCCTC R:GGGCAGATGTTGGTGAGGTA	$(GGA)_5$	1	131	55	JQ307449
BS404	F:TCTTCTCAATCCGCCATTTC R:TCTACTTCCACCTGGCTGCT	$(AGC)_4$	1	103	55	JQ307456
BS448	F:CGTCAGAGGATCTCCGAAAC R:CATTACCAGGGATTGACGCT	$(GGC)_3$	1	177	55	JQ307457
BS486	F:CTACAACGTGCTCAACCGAA R:GCGACCACAAGGAGAAGAAG	$(CTT)_5$	1	278	55	JQ307458
BS520	F:CCTCTCCCTCTTCGTTCTCC R:GGACGTCGACGCTGTTGTTG	$(GCA)_3$	1	273	55	JQ307459
BS526	F:ACAGAAGGAGCTGGTGGAGA R:AGATGATGAGCTGGTCTGGG	$(CCCAAG)_2$	1	125	55	JQ307460
BS529	F:CTCTGCATCACCATCACCAC R:AGAAGACGGCCGTGTCGTAG	$(TCT)_3$	1	133	55	JQ307461
BS534	F:CAGCATCACCGTCAACCAGG R:TAACCACCGCCTCCGCCGTA	$(GGC)_5$	1	474	55	JQ307462
BS544	F: ACTTGGAACGAAAAGAGGGG R: AGTTGATGGACTGGAGCTGG	$(GCG)_5$	1	282	55	JQ307464
2-3D12	F:TGTGACAGCCATAGATATCGGC R:ATCACTCGTTAATATTCCCTACTAGTG	$(GAGT)_3$ $(GAAAA)_2$	1	200	55	EF450749
2-6C3	F:AGCAACCACCAAACCCTTC R:CTCGTCGTCTCCAACCTCTC	$(CT)_{16}$	1	218	55	EF450756
2-6H1	F:ATGATCCCTGCATTCTCGTC R:CGTCGTTTCTGCTTGGATTT	$(CTT)_{23}$	1	160	55	EF450759
3-2A7	F:CTTATGCCTTTCCAGGACGA R:CCTGCACTGCTAATCAACCA	$(ATCT)_6$	1	180	55	EF450750
3-2G2	F:TACAGACGAACCTGGCAGAC R:GCCTACCTCAACTTGCTTGG	$(AAAC)_5$	1	174	55	EF450752
3-4E8	F:ACATGGTAAGAACCAGAATCGG R:TGAATTCGGCACGTCTGGATCC	$(CAAA)_9$	1	241	55	EF450747
3-4F9	F:GCTCAGCTTGTTCTTTTACCCATATC R:TTGCCACCGCCTCTTAACATAC	$(GATT)_6$	1	251	55	EF450748

Ta, PCR annealing temperature; N_A, number of alleles; Size range, size of alleles.

were successfully amplified and generated clear fragments were further used to detect polymorphisms for all the 100 individuals from the five natural populations. They were screened on 8% denaturing polyacrylamide gels and visualized by silver staining with a 100 bp extended DNA ladder as a size standard.

2.2.3. Data Analysis

Genetic diversity of SSR loci including percentage of polymorphic loci (*PPL*), observed heterozygosity (H_O), and expected heterozygosity (*He*) were calculated by using GenAlEx 6.5 [16]. GENEPOP 4.1 [17] was performed to test for deviations from Hardy-Weinberg equilibrium, estimate the Wright's F_{st} and calculate the Wright's fixation index (F_{is}). The genetic relationships among populations were also inferred using the proportions of alleles according to the similarity estimation [18]. A dendrogram was drawn by the unbiased Nei's genetic distance [19] based on the unweighted pair group method with arithmetic averages (UPGMA) using the TFPGA program [20]. Genetix 4.03 software was applied to perform a factorial correspondence analysis (FCA) [21].

3. Results and Discussion

3.1. Levels of Genetic Variation

A total of thirty-seven microsatellite loci were successfully amplified in *B. sylvaticum* var. *breviglume*. Twenty-five of them were deposited at GenBank (Accession Number from JQ307441 to JQ307465) (**Table 2**). Our results showed that seven of them were polymorphic within the five studied populations, while the other thirty were monomorphic. Polymorphic screening found that the number of alleles ranged from 5 to 24 in the five natural populations when using seven polymorphic microsatellite loci (**Table 2**).

The mean number of alleles per locus (*Na*) varied from 2.86 to 3.43, with an average of 3.14, while the effective number of alleles per locus (*Ne*) varied from 2.03 to 2.53 with an average of 2.30. The observed heterozygosities (*Ho*) ranged from 0.029 to 0.220, with an average of 0.105. The expected heterozygosity (*He*) varied from 0.439 to 0.561 with an average of 0.488, while the percentage of polymorphic loci ranged from 85.71% to 100%, with an average of 97.14% (**Table 1**). Among the five studied populations, YY harbored the largest levels of genetic diversity, while TH possessed the lowest genetic variation.

In this study, high level of polymorphisms was detected by the seven SSR loci with the total number of 94 alleles. Levels of allelic diversity (*Na* = 3.14; *Ne* = 2.30), observed and expected heterozygosity within populations (mean *Ho* = 0.105; *He* = 0.488) were detected to be higher than those within 23 invasive populations (*Rs* = 1.403; mean *Ho* = 0.081; *Hs* = 0.212) but almost equal to 25 native populations (*Rs* = 1.871; mean *Ho* = 0.225; *He* = 0.406) [3].

3.2. Population Genetic Structure

Overall, estimates of the expected heterozygosity were found to range from 0.206 (3-2E3) to 0.728 (2-3A1) with an average of 0.488, which were higher than values of observed heterozygosity varied from 0.056 (2-6E6) to 0.217 (2-6E8) with an average of 0.105 (**Table 3**). F_{is} values ranged from 0.628 to 0.885 with an average value of 0.784. Our results indicate that all microsatellite loci have deviated from Hardy-Weinberg expectations and show a deficiency of heterozygotes in the studied populations of the species. The tendency toward heterozygote deficiency suggests the presence of inbreeding within the populations of *B. sylvaticum* var. *breviglume*.

Table 3. Measurements of genetic diversity, genetic differentiation and gene flow among populations of *B. sylvaticum* var. *breviglume* across the seven microsatellite loci.

Locus	*Na*	*Ne*	H_o	H_e	F_{is}	F_{st}	N_m
2-3A1	5.600	3.971	0.128	0.728	0.824	0.136	1.586
2-6E6	3.400	2.129	0.056	0.486	0.885	0.258	0.718
2-6E8	3.800	2.622	0.217	0.584	0.628	0.352	0.459
3-2B2	2.400	2.284	0.092	0.522	0.824	0.328	0.513
3-2E3	1.800	1.325	0.052	0.206	0.750	0.671	0.123
BS537	2.800	2.016	0.121	0.499	0.758	0.266	0.691
BS545	2.200	1.736	0.071	0.392	0.820	0.306	0.567
Mean	3.14	2.30	0.105	0.488	0.784	0.331	0.665

Note: F_{is} deficiencies of heterozygotes relative to Hardy-Weinberg expectations; *Nm*, gene flow estimated from F_{st} ($Nm = 0.25 \times (1 - F_{st})/F_{st}$).

The coefficient of hierarchical F_{st} was observed to range from 0.136 (2-3A1) to 0.671 (3-2E3) with an average value of 0.331. The results suggest that there is a moderate genetic differentiation among the studied populations. Gene flow is regarded as an important cohesive force to hold the geographically separated populations together into a single evolutionary unit, which might result in the reduced genetic differences between populations [22]. In this study, the extent of gene flow (N_m) ranged from 0.123 (3-2E3) to 1.586 (2-3A1) and overall N_m was estimated to equal 0.665. The results indicate the restricted gene flow among the studied populations of *B. sylvaticum* var. *breviglume*.

The UPGMA dendrogram based on the Nei's unbiased genetic distances revealed the existence of genetic differentiation among populations (**Figure 1**). Cluster A included three populations (TH, ML and JZ), the clusters B and C comprised YL and YY, respectively. The result was further supported by three-dimensional factorial correspondence analysis (FCA), indicating a similar genetic relationships of the studied populations. The three-axis variances of 32.21%, 29.13% and 24.15%, respectively, showed the distinction of three clusters (**Figure 2**).

The genetic differentiation among the studied populations (F_{st} = 0.488) was higher than the value detected in native populations of *B. sylvaticum* from Europe, North Africa and the Middle East (F_{st} = 0.441) but lower than that among invasive populations from Oregon and California, USA (F_{st} = 0.493). The F_{is} value (F_{is} = 0.784) detected in natural populations of *B. sylvaticum* var. *breviglume* in this study was higher than the previously reported native (F_{is} = 0.447) and invasive populations (F_{is} = 0.616) of *B. sylvaticum* [3]. The result suggests that the studied populations of *B. sylvaticum* var. *breviglume* from China exhibits high extent of inbreeding.

4. Conclusion

This study has successfully screened thirty-seven microsatellite markers in *B. sylvaticum* var. *breviglume*. The highly discriminatory power of seven polymorphic microsatellite loci suggests that they may be suitable for both the large-scale and fine-scale analyses of population structure of the species. These newly characterized SSR

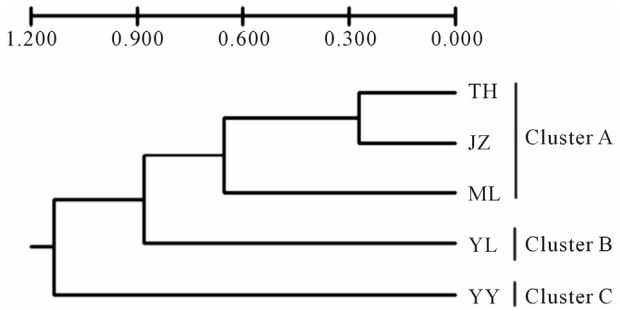

Figure 1. Genetic relationships among populations of *B. sylvaticum* var. *breviglume*. The UPGMA dendrogram was constructed based on the Nei's unbiased genetic distances.

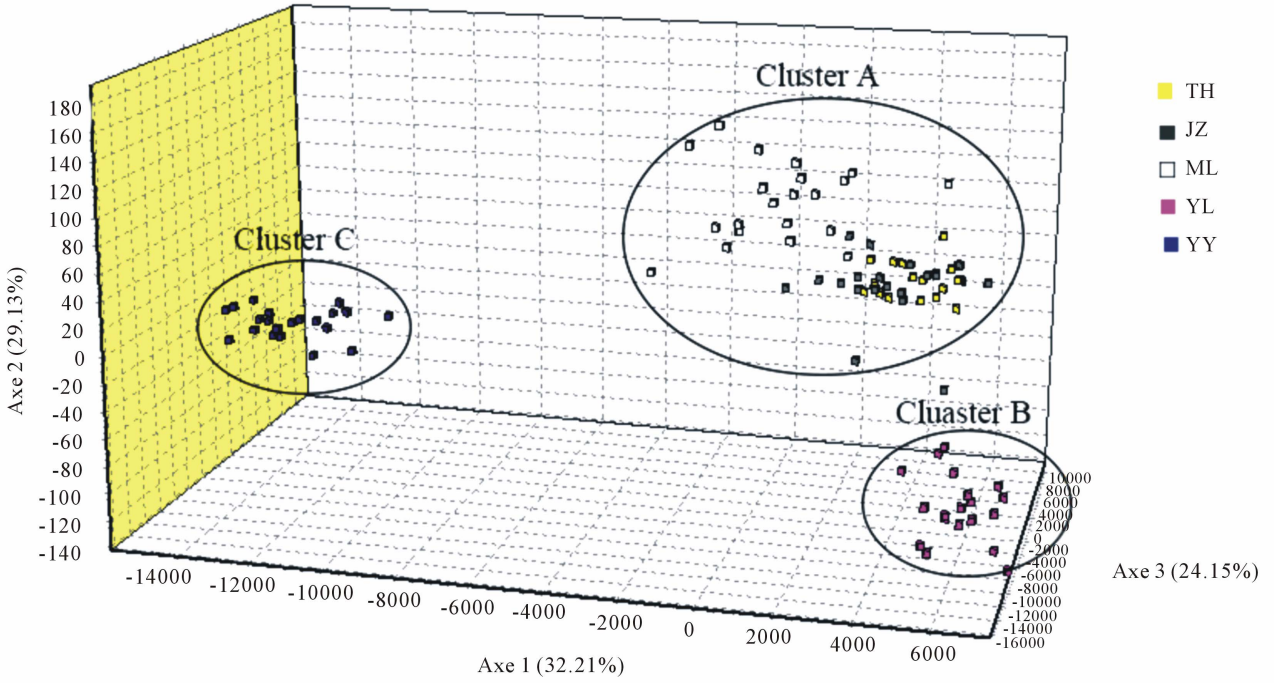

Figure 2. The factorial correspondence analysis (FCA) of 100 individuals of the five natural populations of *B. sylvaticum* var. *breviglume*. The analysis allows reliable distinction of the three clusters of A, B and C.

markers should be useful for the exploration of genetic diversity and evolutionary history of the *B. sylvaticum* var. *breviglume* populations in Yunnan, China.

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