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# Genetic Analyses of Chinese *Cynodon* Accessions by Flow Cytometry and AFLP Markers

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## ABSTRACT

**Bermudagrass [*Cynodon dactylon* (L.) Pers.] is widely distributed in China, but little information exists on genetic diversity within the germplasm pool. This study was conducted to assess variations in ploidy and amplified fragment length polymorphisms (AFLPs) among *Cynodon* accessions collected from 11 Chinese provinces. Flow cytometry and AFLP analyses were performed on 132 and 119 Chinese accessions, respectively. Four ploidy cytotypes were found among the Chinese accessions. Tetraploid ($2n = 4x = 36$) accessions were most prevalent (88%), with nuclear genome sizes ranging from 1.96 to 2.30 pg/2C nucleus$^{-1}$. Seven hexaploid ($2n = 6x = 54$), three pentaploid ($2n = 5x = 45$), and six triploid ($2n = 3x = 27$) accessions had respective nuclear genome size of 2.90 to 3.13, 2.37 to 2.49, and 1.55 to 1.65 pg/2C nucleus$^{-1}$. The accessions were grouped into five clusters based on 466 polymorphic AFLP bands. Genetic similarity coefficients (GSCs) of two clusters containing 'Tifway' and 'Tifgreen' ranged from 0.97 to 0.99, suggesting the triploid plants most probably were introduced cultivars from the USA. Within the Chinese indigenous accessions, GSC ranged from 0.65 to 0.99. Tetraploid genotypes had the greatest genetic variation with GSC ranging from 0.69 to 0.99, while pentaploids had the least with GSC ranging from 0.95 to 0.98. Genetic differentiation among the later three ploidy levels is evident. Fully sampling the genetic diversity of *Cynodon* in China will require more comprehensive collection throughout its distribution.**

Grasses belonging to the genus *Cynodon* L. C. Rich. occur in relative abundance in the southern half of China and are sparsely distributed in northern warm temperate humid regions (Anonymous, 1990; Abulaiti et al., 1998). Three *Cynodon* taxa, *C. arcuatus* J.S. Presl ex C.B. Presl, *C. dactylon* (L.) Pers. var. *dactylon*, and *C. dactylon* var. *biflorus* Merino, are listed in *Chinese Floral Acta* (Anonymous, 1990). The taxonomic revision of *Cynodon* by J.R. Harlan and colleagues included nine species and 10 varieties, but did not include *C. dactylon* var. *biflorus* (Harlan et al., 1970a; de Wet and Harlan, 1970). Harlan et al. (1970a) provided a description of *C. arcuatus* and indicated that it was easily distinguished and genetically isolated from other *Cynodon* species. *C. dactylon* var. *dactylon* is the most widely distributed and genetically variable of all *Cynodon* species (Harlan and de Wet, 1969; Harlan et al., 1970a). It is found on all continents and larger ocean islands between about 45° N and S latitudes. Its uses as livestock fodder, turf, and soil stabilization and remediation make it the most economically important of the *Cynodon* species (Burton, 1947; Harlan, 1970; Harlan et al., 1970b; Taliaferro, 1995, 2003).

Although the widespread occurrence of *Cynodon* in China is well documented, there is little information on kinds and magnitudes of genetic variation within the indigenous Chinese *Cynodon* germplasm pool. In 1974, G.W. Burton collected an unusually dark green *C. dactylon* plant from a Shanghai lawn that was later determined to be a hexaploid ($2n = 6x = 54$ chromosomes) and released as 'Tifton 10' (Hanna et al., 1990). In addition to Tifton10, only a few hexaploid *Cynodon* plants have been reported (Hurcombe, 1947; Moffett and Hurcombe, 1949; Powell et al., 1968; Felder, 1967; Malik and Tripathi, 1968; Johnston, 1975). The basic chromosome number of *Cynodon* is nine and the predominant cytotypes are diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) (Forbes and Burton, 1963; Harlan et al., 1970c; de Wet and Harlan, 1971; de Silva and Snaydon, 1995). Triploid ($2n = 3x = 27$) *Cynodon* plants from intra- and interspecific natural and artificial hybridizations are relatively common. Rare pentaploid plants have been reported from interspecific artificial hybridization (Johnston, 1975; Burton et al., 1993).

The PCR-based DNA fingerprinting techniques based on the analysis of information-rich nucleic acid molecules have been used in studies of genetic diversity, relatedness, phylogeny, and in identifying off-types of cultivars in *Cynodon* (Caetano-Anolles, 1998a). A number of researchers have employed randomly amplified polymorphic DNA (RAPD) (Roodt et al., 2002) and DNA amplified fingerprinting (DAF) procedures (Caetano-Anolles et al., 1995; Caetano-Anolles et al., 1997; Ho et al., 1997; Assefa et al., 1998; Anderson et al., 2001). Recently, AFLP (Vos et al., 1995) has been used in *Cynodon* to differentiate bermudagrass genotypes (Zhang et al., 1999), to detect the genetic diversity among forage bermudagrass cultivars (Karaca et al., 2002), and to quantify the genetic variation of *C. transvaalensis* and its relatedness to hexaploid *C. dactylon* (Wu et al., 2005).

The objectives of this study were to (i) determine the ploidy of 132 *Cynodon* accessions collected in 11 provinces of China, and (ii) quantify the genetic variation and relatedness of the accessions within and among different ploidy levels based on AFLP markers.

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**Abbreviations:** AFLP, amplified fragment length polymorphism; DAF, DNA amplified fingerprinting; GSC, genetic similarity coefficient; PIC, polymorphic information content; UPGMA, unweighted pair group mean algorithm.

**Table 1. Identification, source, nuclear DNA content, and inferred ploidy of 132 Chinese *Cynodon* accessions and four commercial *Cynodon* cultivars.**

| Identification | Origin or reference | DNA content mean ± SD | Inferred ploidy (2*n*) |
|---|---|---|---|
| | | pg/2C | |
| A12253 | Sichuan | 2.15 ± 0.07 | 36 |
| A12254 | Sichuan | 2.05 ± 0.06 | 36 |
| A12255 | Sichuan | 2.04 ± 0.03 | 36 |
| A12256 | Shanghai | 1.64 ± 0.03 | 27† |
| A12257 | Chongqing | 1.96 ± 0.07 | 36 |
| A12258 | Chongqing | 2.09 ± 0.04 | 36 |
| A12259 | Sichuan | 2.01 ± 0.04 | 36 |
| A12260 | Sichuan | 1.65 ± 0.01 | 27† |
| A12261 | Sichuan | 1.55 ± 0.01 | 27 |
| A12262 | Yunnan | 2.09 ± 0.03 | 36 |
| A12263 | Yunnan | 2.03 ± 0.05 | 36 |
| A12264 | Sichuan | 2.07 ± 0.01 | 36 |
| A12265 | Sichuan | 2.02 ± 0.02 | 36 |
| A12266 | Sichuan | 2.17 ± 0.04 | 36 |
| A12267 | Sichuan | 2.10 ± 0.01 | 36 |
| A12268 | Sichuan | 2.07 ± 0.04 | 36 |
| A12269 | Sichuan | 2.05 ± 0.02 | 36 |
| A12270 | Sichuan | 2.03 ± 0.04 | 36 |
| A12271 | Sichuan | 2.05 ± 0.03 | 36 |
| A12272 | Sichuan | 1.61 ± 0.01 | 27† |
| A12273 | Sichuan | 2.09 ± 0.06 | 36 |
| A12274 | Sichuan | 2.11 ± 0.01 | 36 |
| A12275 | Sichuan | 1.99 ± 0.04 | 36 |
| A12276 | Sichuan | 2.22 ± 0.05 | 36 |
| A12277 | Sichuan | 2.02 ± 0.02 | 36 |
| A12278 | Sichuan | 2.01 ± 0.07 | 36 |
| A12279 | Sichuan | 2.06 ± 0.04 | 36 |
| A12280 | Sichuan | 2.03 ± 0.05 | 36 |
| A12281 | Sichuan | 2.20 ± 0.03 | 36 |
| A12282 | Sichuan | 1.55 ± 0.03 | 27† |
| A12283 | Sichuan | 2.15 ± 0.04 | 36 |
| A12284 | Sichuan | 2.12 ± 0.04 | 36 |
| A12285 | Sichuan | 2.08 ± 0.07 | 36 |
| A12286 | Sichuan | 2.03 ± 0.05 | 36 |
| A12287 | Sichuan | 2.11 ± 0.05 | 36 |
| A12288 | Sichuan | 2.00 ± 0.06 | 36 |
| A12289 | Sichuan | 2.10 ± 0.04 | 36 |
| A12290 | Sichuan | 2.08 ± 0.06 | 36 |
| A12291 | Sichuan | 2.11 ± 0.05 | 36 |
| A12292 | Chongqing | 2.04 ± 0.06 | 36 |
| A12293 | Chongqing | 2.10 ± 0.05 | 36 |
| A12294 | Sichuan | 2.12 ± 0.05 | 36 |
| A12295 | Sichuan | 2.11 ± 0.04 | 36 |
| A12337 | Sichuan | 2.05 ± 0.05 | 36 |
| A12338 | Sichuan | 2.10 ± 0.05 | 36 |
| A12339 | Sichuan | 2.30 ± 0.03 | 36 |
| A12340 | Sichuan | 2.14 ± 0.06 | 36 |
| A12341 | Sichuan | 2.01 ± 0.05 | 36 |
| A12342 | Sichuan | 2.06 ± 0.06 | 36 |
| A12343 | Sichuan | 2.17 ± 0.07 | 36 |
| A12344 | Sichuan | 2.05 ± 0.02 | 36 |
| A12345 | Sichuan | 2.12 ± 0.02 | 36 |
| A12346 | Sichuan | 2.12 ± 0.01 | 36 |
| A12347 | Sichuan | 2.12 ± 0.03 | 36 |
| A12348 | Hainan | 2.45 ± 0.04 | 45† |
| A12349 | Hainan | 2.16 ± 0.07 | 36 |
| A12350 | Guangdong | 2.14 ± 0.07 | 36 |
| A12351 | Hainan | 2.08 ± 0.02 | 36 |
| A12352 | Hainan | 2.37 ± 0.02 | 45† |
| A12353 | Guangdong | 2.04 ± 0.05 | 36 |
| A12354 | Guangdong | 2.03 ± 0.03 | 36 |
| A12355 | Zhejiang | 2.13 ± 0.06 | 36 |
| A12356 | Zhejiang | 3.13 ± 0.05 | 54† |
| A12357 | Jiangsu | 2.02 ± 0.07 | 36 |
| A12358 | Jiangsu | 2.98 ± 0.05 | 54† |
| A12359 | Jiangsu | 2.02 ± 0.06 | 36 |
| A12360 | Jiangsu | 2.90 ± 0.08 | 54† |
| A12361 | Jiangsu | 2.01 ± 0.07 | 36 |
| A12296 | Sichuan | 2.13 ± 0.05 | 36 |
| A12297 | Sichuan | 2.08 ± 0.02 | 36 |
| A12298 | Chongqing | 2.06 ± 0.06 | 36 |
| A12299 | Chongqing | 2.22 ± 0.05 | 36 |
| A12300 | Sichuan | 2.04 ± 0.07 | 36 |
| A12301 | Sichuan | 2.05 ± 0.04 | 36 |
| A12302 | Sichuan | 2.02 ± 0.05 | 36 |
| A12303 | Sichuan | 2.04 ± 0.04 | 36 |
| A12304 | Sichuan | 2.09 ± 0.05 | 36 |
| A12305 | Sichuan | 2.08 ± 0.04 | 36 |
| A12306 | Sichuan | 2.05 ± 0.03 | 36 |
| A12307 | Sichuan | 2.13 ± 0.06 | 36 |
| A12308 | Sichuan | 2.07 ± 0.05 | 36 |
| A12309 | Sichuan | 2.12 ± 0.02 | 36 |
| A12310 | Sichuan | 2.07 ± 0.03 | 36 |
| A12311 | Sichuan | 2.13 ± 0.05 | 36 |
| A12312 | Sichuan | 2.08 ± 0.05 | 36 |
| A12313 | Sichuan | 2.04 ± 0.05 | 36 |
| A12314 | Chongqing | 2.06 ± 0.06 | 36 |
| A12315 | Shanghai | 2.15 ± 0.06 | 36 |
| A12316 | Shanghai | 2.09 ± 0.03 | 36 |
| A12317 | Shanghai | 3.12 ± 0.05 | 54† |
| A12318 | Shanghai | 2.99 ± 0.05 | 54† |
| A12319 | Shanghai | 3.08 ± 0.06 | 54† |
| A12320 | Sichuan | 3.15 ± 0.04 | 54† |
| A12321 | Sichuan | 2.03 ± 0.05 | 36 |
| A12322 | Sichuan | 2.17 ± 0.04 | 36 |
| A12323 | Sichuan | 2.00 ± 0.04 | 36 |
| A12324 | Chongqing | 2.08 ± 0.07 | 36 |
| A12325 | Sichuan | 2.15 ± 0.02 | 36 |
| A12326 | Yunnan | 1.99 ± 0.06 | 36 |
| A12327 | Sichuan | 2.02 ± 0.06 | 36 |
| A12328 | Sichuan | 2.07 ± 0.08 | 36 |
| A12329 | Sichuan | 2.26 ± 0.03 | 36 |
| A12330 | Sichuan | 2.07 ± 0.05 | 36 |
| A12331 | Sichuan | 2.07 ± 0.07 | 36 |
| A12332 | Sichuan | 2.06 ± 0.06 | 36 |
| A12333 | Sichuan | 2.00 ± 0.05 | 36 |
| A12334 | Sichuan | 2.21 ± 0.02 | 36 |
| A12335 | Sichuan | 2.09 ± 0.05 | 36 |
| A12336 | Sichuan | 2.05 ± 0.03 | 36 |
| A12362 | Fujian | 2.03 ± 0.06 | 36 |
| A12363 | Jiangsu | 2.02 ± 0.06 | 36 |
| A12364 | Zhejiang | 2.07 ± 0.05 | 36 |
| A12365 | Fujian | 2.49 ± 0.03 | 45† |
| A12366 | Fujian | 2.12 ± 0.04 | 36 |
| A12367 | Shandong | 2.09 ± 0.07 | 36 |
| A12368 | Beijing | 2.01 ± 0.01 | 36 |
| A12369 | Hainan | 1.62 ± 0.02 | 27 |
| A12370‡ | Hainan | 2.15 ± 0.05 | 36 |
| A12371 | Hainan | 2.10 ± 0.05 | 36 |
| A12372 | Guangdong | 2.00 ± 0.02 | 36 |
| 93–138‡ | Yunnan | 2.02 ± 0.06 | 36 |
| 93–139‡ | Yunnan | 2.05 ± 0.06 | 36 |
| 93–140‡ | Yunnan | 2.05 ± 0.06 | 36 |
| 93–141‡ | Yunnan | 2.04 ± 0.02 | 36 |
| 93–142‡ | Yunnan | 2.11 ± 0.05 | 36 |
| 93–143‡ | Yunnan | 2.04 ± 0.04 | 36 |
| 93–144‡ | Yunnan | 2.02 ± 0.06 | 36 |
| 93–145‡ | Yunnan | 2.05 ± 0.03 | 36 |
| 93–146‡ | Yunnan | 2.25 ± 0.05 | 36 |
| 93–147‡ | Yunnan | 2.03 ± 0.05 | 36 |
| 93–148‡ | Yunnan | 2.10 ± 0.05 | 36 |
| 93–149‡ | Yunnan | 2.08 ± 0.03 | 36 |
| Tifton 10 | Hanna et al., 1990 | 2.90 ± 0.08 | 54 |
| Tifgreen | Hanson, 1972 | 1.61 ± 0.03 | 27 |
| Uganda‡ | Hanson, 1972 | 1.05 ± 0.02 | 18 |
| Tifway | Hanson, 1972 | 1.56 ± 0.05 | 27 |

† Chromosomes of the accession was determined or confirmed by cytological observations of 5 to 16 cells.
‡ The accession was not used in AFLP analysis.

## MATERIALS AND METHODS

### Plant Materials

Plant materials consisted of 136 *Cynodon* clonal accessions including 132 accessions collected in China and four U.S. commercial cultivars with known chromosome number and nuclear DNA content (Table 1). The Chinese *Cynodon* accessions were collected from 11 provinces ranging from

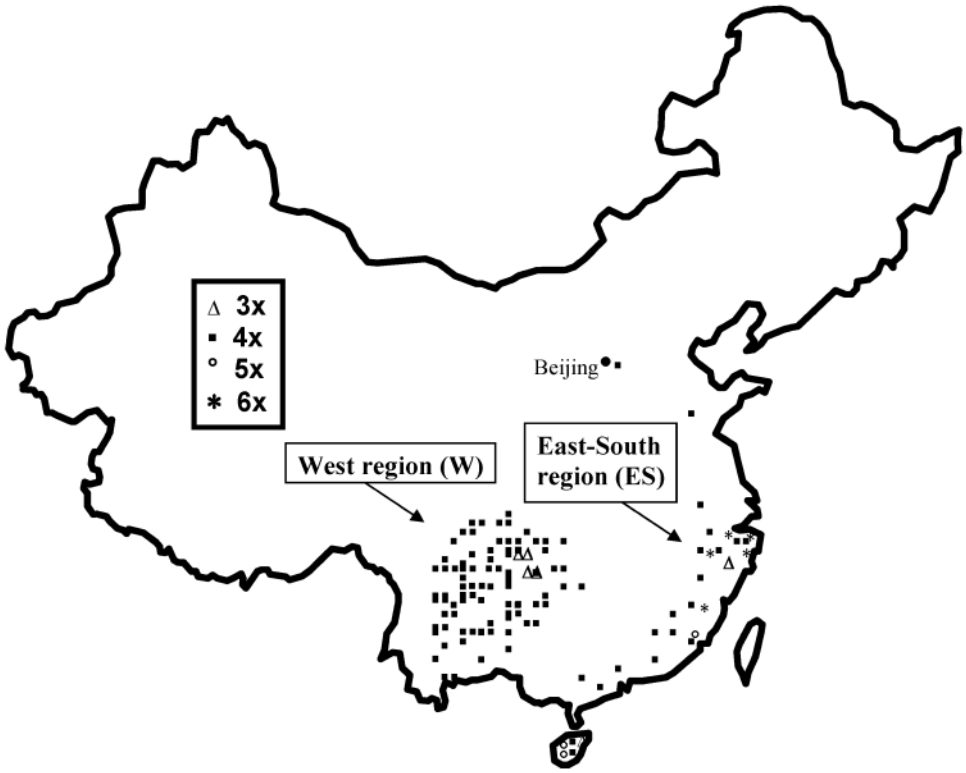


**Fig. 1. A map showing the Chinese *Cynodon* collection locations and their ploidy.**

tropical Hainan Island to the temperate climatic region around Beijing (Fig. 1). All indigenous Chinese accessions were determined to be *C. dactylon* based on morphological characteristics, as described by Harlan et al. (1970a) and Clayton et al. (2005). Distinguishing characteristics of *C. dactylon* include racemes arranged in one whorl on inflorescences and subequal glumes at least 3/4 the length of spikelets.

The plants were grown in a greenhouse at the Agronomy Research Station, Oklahoma State University, Stillwater, OK, in 15-cm-diam. pots containing Metro-Mix 250 growing medium (Scotts-Sierra Horticultural Products Co., Marysville, OH). They were watered daily and fertilized biweekly with M-77 Peat-lite Special water-soluble fertilizer (Scotts-Sierra Horticultural Products Co., Marysville, OH). All plants were actively growing and healthy at the time of the sampling.

## Flow Cytometry

Flow cytometry procedures described by Taliaferro et al. (1997b) were used to measure nuclear DNA content on the 136 accessions. Flow cytometry analyses of prepared materials were conducted on a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA) with an argon laser emitting at 488 nm for excitation of propidium iodide at the Flow Cytometry Laboratory of Oklahoma State University. The mean nuclear DNA content of each plant sample, measured in picograms, was based on 5000 scanned nuclei. Channel catfish (*Ictalurus punctatus*) blood cells with a nuclear DNA content of 5.67 pg/2C were used as internal standards (Taliaferro et al., 1997b). For every plant sample, at least three measurements (replicates) were obtained in three different days. Sample DNA content was calculated by dividing the mean value of sample channel by the mean value of internal standard channel, then multiplying the result by the DNA content of the internal standard. The ploidy levels of the Chinese accessions were inferred by comparing sample DNA content to the previously reported range of respective ploidy levels (Taliaferro et al., 1997b; Arumuganathan et al., 1999).

## Cytology

Somatic chromosome number was determined for all accessions having DNA content values outside the reported range for respective ploidy levels (Taliaferro et al., 1997b; Arumuganathan et al., 1999). Additionally, somatic chromosome number was determined for selected accessions having DNA content within the reported range to confirm the accuracy of the flow cytometry data. Somatic chromosome counts were determined using conventional squashes of shoot tip somatic cells under a light microscope equipped with a digital camera system. The somatic cell squashes were prepared from shoot apical meristem tissues as described by Powell (1968) with minor modifications. Briefly, actively growing shoot tips were stripped, and the stripped shoot tips were cut longitudinally to expose the apical meristem. As a pretreatment to shorten chromosomes, the split shoot tips were placed into a saturated solution of monobromonaphthalene for 2 h at ambient room temperature. Then the pretreated shoot tips were fixed in 3:1 ethanol–acetic acid solution for 24 h at room temperature. The fixed shoot tips were washed in flowing deionized water for 10 min, then digested in cytolase (PCL5, DSM Food Specialties, Charlotte, NC) solution at 37°C for 40 to 70 min. Chromosomes were stained with acetocarmine.

## AFLP and Marker Data Analysis

In AFLP analysis, 119 Chinese *Cynodon* accessions plus Tifway, Tifgreen, and Tifton 10 (see Table 1 and its footnotes) were used, while the other 13 Chinese accessions were not included because of a space limitation in gel electrophoresis.

DNA samples were isolated from fresh leaf tissues of the *Cynodon* plants with DNeasy plant mini kit from QIAGEN, Inc. (Valencia, CA). The AFLP analysis was performed as described by Vos et al. (1995) with a few minor modifications (Bai et al., 1999). The AFLP procedure details followed Wu et al. (2005).

The AFLP electrophoresis bands were visually scored twice as present (1), absent (0), or ambiguous (9) for each accession. Data of polymorphic bands were compiled for each *Cynodon* accession in a data matrix and analyzed using NTSYS-pc (Numerical Taxonomy System) v. 2.0 (Exeter Software, Setauket, NY). Genetic similarity coefficients of pair-wise comparisons among the *Cynodon* accessions were computed as simple matching coefficients within the SIMQUAL module. Polymorphic information content (PIC) indicating the ability to distinguish between genotypes for each primer combination was calculated as expected heterozygosity for polymorphic bands following Powell et al. (1996). Cluster analysis was performed according to the unweighted pair group mean algorithm (UPGMA) within the SAHN module of the NTSYS-pc program. Bootstrap analysis (250 replications) of the data matrix was performed to test the clusters robustness with FreeTree program according to the program manual (Hampl et al., 2001). A principal coordinate analysis to construct a two-dimensional array of eigenvectors was performed using the DCENTER module of the NTSYS-pc program. Comparisons of the average GSCs among three ploidy levels (4*x*, 5*x*, and 6*x*) of the Chinese *Cynodon* accessions were performed using the program MANTEL-STRUCT with an asymptotic solution (Miller, 1999).

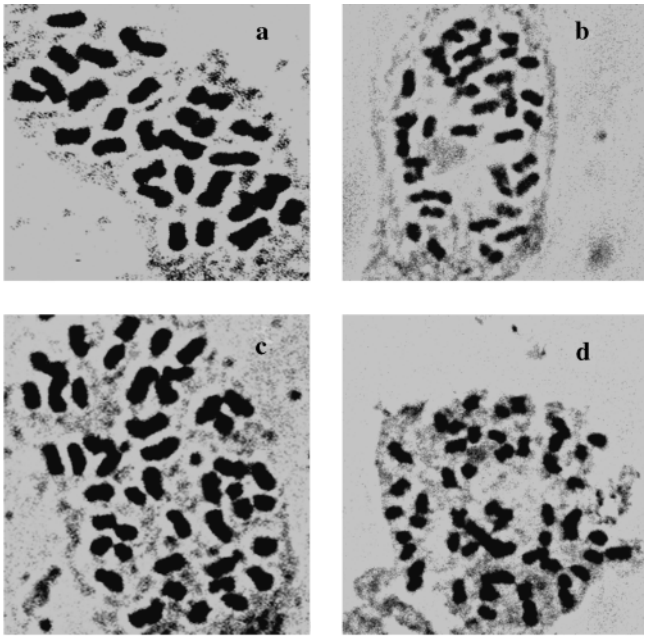


**Fig. 2. Photomicrographs of somatic chromosomes of the four observed ploidy levels in Chinese *Cynodon* accessions. (a) A12260, 2*n* = 3*x* = 27; (b) A12257, 2*n* = 4*x* = 36; (c) A12348, 2*n* = 5*x* = 45; (d) A12319, 2*n* = 6*x* = 54.**

## RESULTS

### Ploidy Determination

The nuclear DNA contents of the 132 Chinese *Cynodon* accessions and four commercial cultivars are presented in Table 1. The standard deviations of DNA content measurements ranged from 0.01 to 0.08 pg/2C nucleus$^{-1}$, demonstrating that flow cytometry was very precise. These results are consistent with previous reports regarding the precision of flow cytometry in *Cynodon* plants (Taliaferro et al., 1997b; Arumuganathan et al., 1999). The DNA contents of Tifton 10, Tifway, Tifgreen, and Uganda, measured along with the Chinese *Cynodon* accessions to verify the results, were consistent with the results reported by Taliaferro et al. (1997b) and Arumuganathan et al. (1999).

Nuclear genome size among the 132 Chinese accessions ranged from 1.55 to 3.15 pg/2C nucleus$^{-1}$ (Table 1). On the basis of previously reported data (Taliaferro et al., 1997b; Arumuganathan et al., 1999), diploid, triploid, tetraploid, and hexaploid cytotypes were indicated by respective nuclear genome sizes of 1.03 to 1.14, 1.37 to 1.62, 1.95 to 2.36, and 2.64 to 2.93 pg DNA 2C$^{-1}$ per nucleus. On the basis of these ranges, four accessions were classified as triploid, 116 accessions as tetraploid, and one accession as hexaploid (see inferred ploidy in Table 1).

Eleven accessions had genome sizes outside the previously reported ranges. Somatic chromosome counts determined for these 11 accessions indicated two triploid (A12256 and A12260), three pentaploids (A12348, A12352, and A12365), and six hexaploids (A12317,

**Table 2. Numbers of total bands and polymorphic bands, percentage polymorphic bands, and polymorphic information content (PIC) for each of 13 AFLP selective primer pairs.**

| Selective amplification primer pairs | Total bands | Polymorphic bands | % polymorphic bands | PIC |
|---|---|---|---|---|
| eAAC/mCAG† | 63 | 25 | 39.68 | 0.16 |
| eAAC/mGAC | 40 | 17 | 42.50 | 0.20 |
| eACT/mCAC | 49 | 42 | 85.71 | 0.19 |
| eACT/mCAG | 61 | 45 | 73.77 | 0.15 |
| eACT/mGAC | 62 | 38 | 61.29 | 0.15 |
| eACT/mCTG | 63 | 46 | 86.79 | 0.19 |
| eAGT/mCAA | 62 | 27 | 43.55 | 0.24 |
| eAGT/mCAC | 72 | 61 | 84.72 | 0.23 |
| eAGT/mCAG | 58 | 37 | 63.79 | 0.21 |
| eAGT/mCAT | 69 | 41 | 59.42 | 0.29 |
| eAGT/mGAC | 53 | 25 | 47.17 | 0.26 |
| eAGT/mCTG | 76 | 45 | 59.21 | 0.27 |
| eGCT/mCAG | 35 | 17 | 48.57 | 0.20 |
| Total | 763 | 466 | | |
| Average | 58.69 ± 11.38 | 35.85 ± 12.40 | 61.07 | 0.21 ± 0.05 |

† e is the preamplification primer sequence for *Eco*RI site (5-GACTGCGTACCAATTC) without any selective nucleotides and m is the preamplification primer sequence for *Mse*I site (5-GATGAGTCCTGAGTAA).

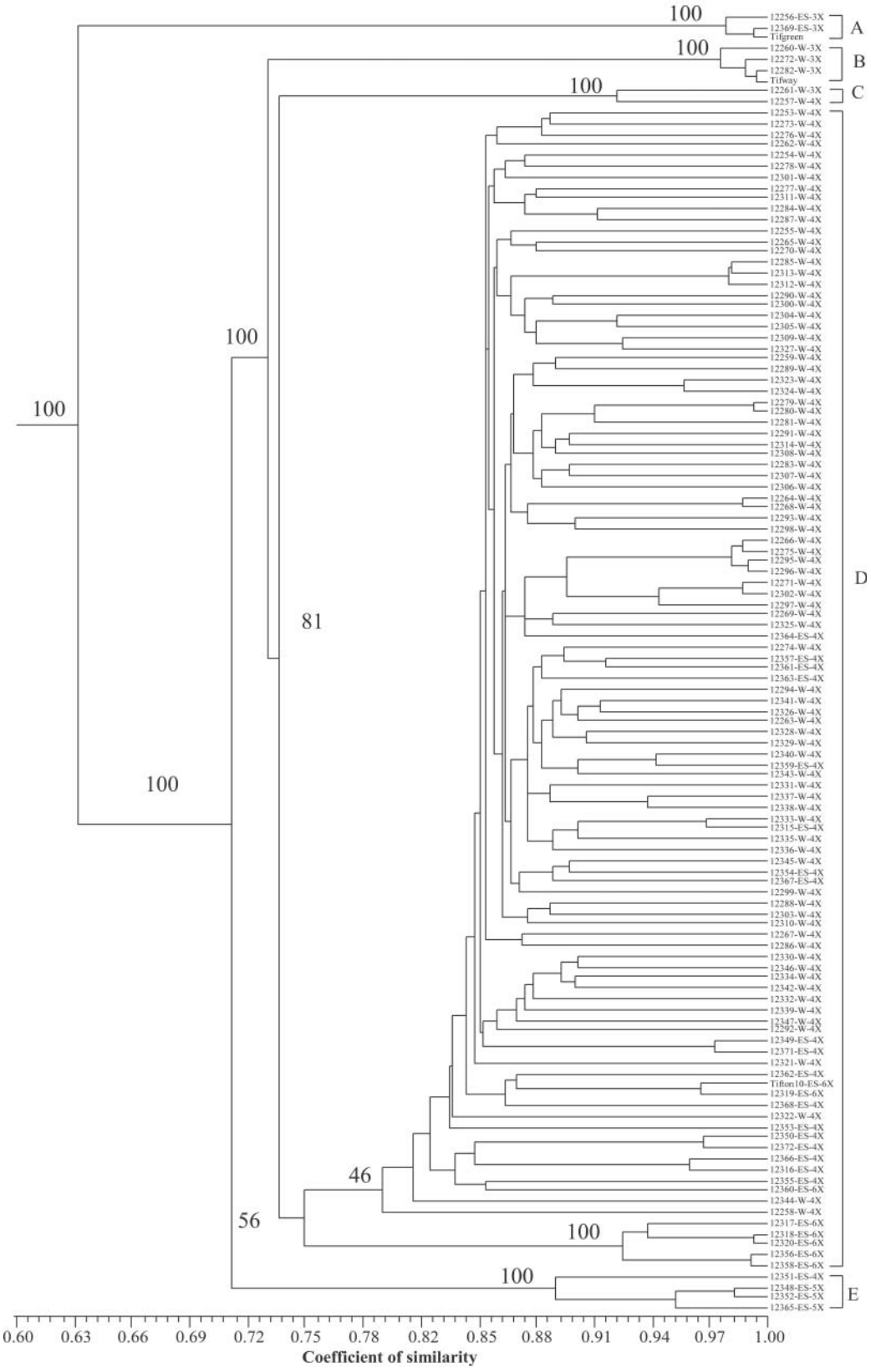


**Fig. 3. Dendrogram of 119 Chinese *Cynodon* accessions plus Tifway, Tifgreen and Tifton 10 produced by unweighted pair group mean algorithm clustering method based on the genetic similarity matrix derived from 466 AFLP markers. Bootstrap values are displayed.**

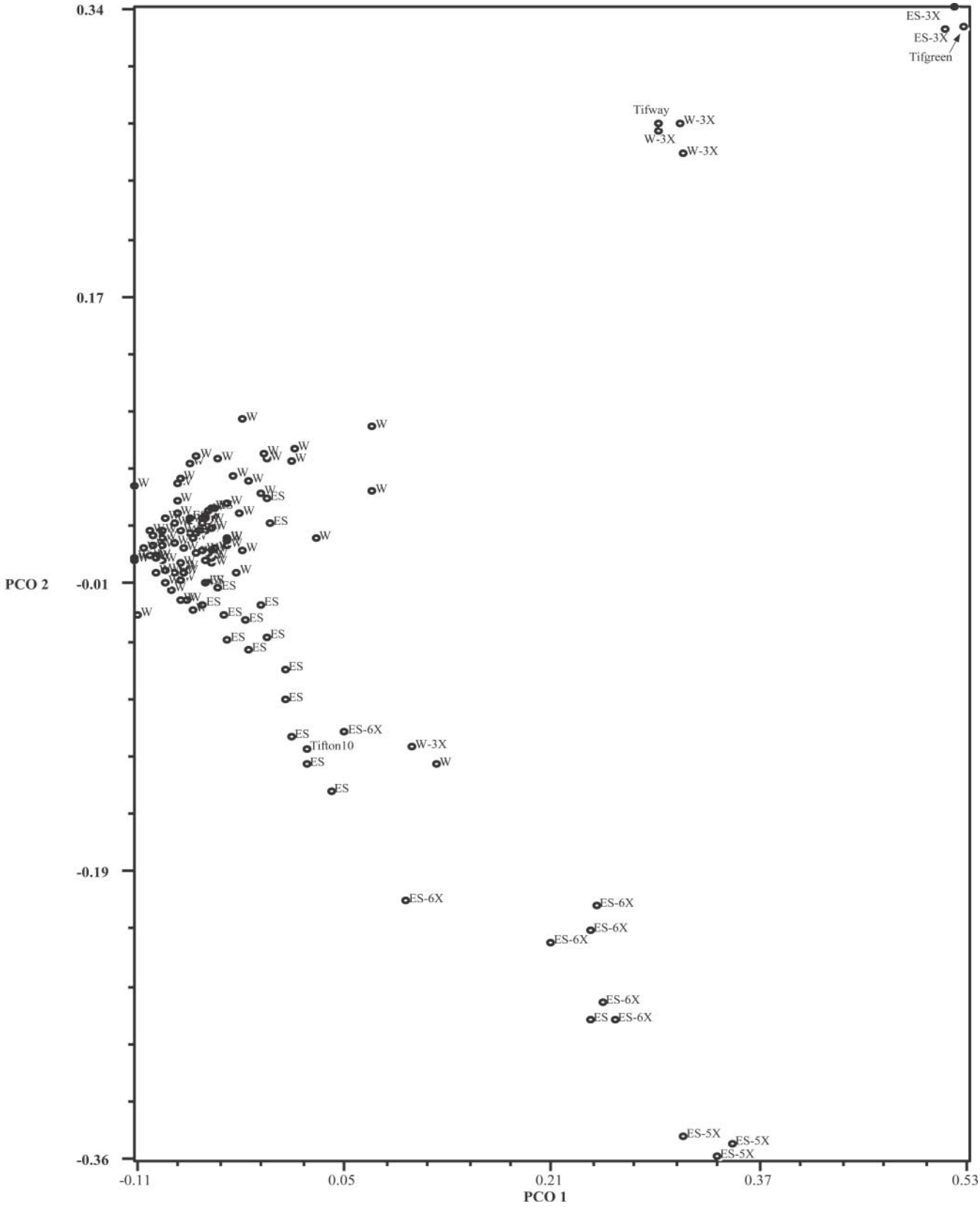


**Fig. 4. Principal coordinate plot indicating variation pattern and geographic origin for the 119 Chinese *Cynodon* accessions and three cultivars using 466 AFLP markers. Notations: W, E, and S stand for the west, east, and south regions, respectively. Ploidy level is also indicated if other than 4*x*.**

A12318, A12319, A12320, A12356, and A12358) (Table 1 and Fig. 2). Somatic chromosome counts for A12272, A12282, A12257, and A12360 confirmed that the three inferred ploidy levels based on nuclear DNA contents were correct (Table 1).

## AFLP Genetic Diversity and Relatedness

Thirteen AFLP selective amplification primer combinations produced a total of 763 bands among the 122 *Cynodon* genotypes, with an average of 58.7 ± 11.4 bands per primer combination (Table 2). Of the 763 bands scored, 466 (61.1%) were polymorphic, with an average of 35.9 ± 12.4 polymorphic bands per primer combination. The primer combinations eAGT/mCAC and eGCT/mCAG amplified the largest (61) and smallest (35) numbers of total bands and polymorphic bands per gel, respectively (Table 2). The average PIC of the 13 primer combinations was 0.21, ranging from 0.15 (eACT/mCAG and eACT/mGAC) to 0.29 (eAGT/mCAT) (Table 2).

The genetic diversity was relatively high among the accessions in this study. The GSCs among the 122 accessions ranged from 0.55 to 0.99. The lowest GSC (0.55) was between Tifgreen and 12351, and Tifgreen and 12261. Accessions 12351 and 12261 were from China. The highest GSC was 0.99, detected between accessions 12279 and 12280. The two accessions were collected from very close sites in Aba, Sichuan Province of China.

Cluster analysis based on the GSC separated the 122 *Cynodon* accessions into five distinct major groups having bootstrap values of 81% or more: A, B, C, D, and E (Fig. 3). The variation patterns and groupings of the *Cynodon* accessions appear to be associated with their ploidy level and geographic origin. Cluster A contains Tifgreen and two triploid accessions (Fig. 3). The GSC among the three accessions ranged from 0.97 to 0.99, indicating that they are genetically very similar. Cluster B consists of Tifway, 12260, 12272, and 12282, with GSC ranging from 0.97 to 0.99 and averaging 0.98 ± 0.01. Cluster C contains only accessions 12261 and 12257. Interestingly, accession 12261 is a triploid collected from Neijiang, Sichuan. Accession 12257 is a tetraploid from Tongjiaxi, Chongqing. The genetic similarity of pair-wise comparison between the two accessions is 0.92.

Cluster D contains 109 accessions making it the largest group. The cluster contains the majority of the tetraploid cytotypes and all of the hexaploid cytotypes of the Chinese accessions (Fig. 3). The GSC in the cluster averaged 0.82 ± 0.05, and ranged from 0.69 to 0.99. The eight hexaploid cytotypes were separated into three subgroups. One subgroup contained 12317, 12318, 12320, 12356, and 12358, with a bootstrap value of 100%. Accessions 12317 and 12318 were collected from Shanghai, and 12356 and 12358 from Zhejiang and Jiangsu, respectively. Field notes indicated that accession 12320 was a fine-textured plant from Hongya, Sichuan, but the plant used in this study had a relatively coarse texture, suggesting that it was not the true 12320 accession. Mechanical mixture of the two accessions may have occurred because the original nursery plots of the two accessions were adjacent to each other. The GSC between 12318 and 12320 was 0.99, further suggesting that the accession identified as 12320 in this study was a contaminant. The other three hexaploid genotypes (Tifton10, 12319, and 12360) clustered into two subgroups. Tifton10 and 12319 were from Shanghai, and 12360 from Jiangsu. In cluster D, 101 of the tetraploid genotypes scatter into various subgroupings (Fig. 3). Collectively, the accessions from the same geographic locations or nearby regions tended to have higher genetic similarity and to cluster into the same subgroups or neighboring subgroups. As shown in Fig. 3, most accessions that originated in the western provinces, such as Sichuan, Chongqing, and Yunnan, were included in the same subgroupings or in neighboring subgroupings, and most accessions from eastern and southern provinces, including Shanghai, Jiangsu, Zhejiang, Fujian, Guangdong, and Hainan, tended to group into the same or adjacent groupings. However, seven accessions (12364, 12357, 12361, 12363, 12354, 12367, and 12359) from eastern and southern regions formed subgroupings with accessions from west regions, while two accessions (12344 and 12258) from western regions formed subgroupings closer to groupings containing predominantly eastern and southern origin accessions.

Cluster E contains one tetraploid and three pentaploid accessions (Fig. 3). Two pentaploid accessions 12348 and 12352 and the tetraploid accession 12351 were collected from Xinlong, Wanquanhe, and Haikou in Hainan Island. Another pentaploid accession 12365 originated in Minghou, Fujian. Among the pentaploid genotypes, GSCs averaged 0.96 ± 0.01, ranging from 0.95 to 0.98, indicating very low genetic diversity among the pentaploid accessions.

Principal coordinate analysis clearly indicated that Tifgreen and Tifway, and the accessions clustering with them, were widely separated from the Chinese accessions (Fig. 4). Both Tifgreen and Tifway are interspecific $F_1$ hybrids from tetraploid *C. dactylon* by diploid *C. transvaalensis* crosses with parents of African origin. Among the Chinese accessions, the pentaploid genotypes separated from tetraploid and hexaploid accessions (Fig. 4). However, the hexaploid accessions were grouped closer to or together with the tetraploid accessions from closer geographic regions. Mantel tests of the average GSCs (Table 3) among the ploidy levels

**Table 3. Genetic similarity coefficients within and among three ploidy levels of Chinese *Cynodon* accessions.**

| | | Genetic similarity coefficient | | | |
|---|---|---|---|---|---|
| | | | Among ploidy | | |
| Ploidy | No. of accessions | Within ploidy (Range) | 4*x* | 5*x* | 6*x* |
| 4*x* | 103 | 0.80 (0.69–0.99) | 1.00 | 0.71** | 0.78** |
| 5*x* | 3 | 0.96 (0.95–0.98) | | 1.00 | 0.73** |
| 6*x* | 8† | 0.85 (0.77–0.99) | | | 1.00 |

** Indicating significant difference between ploidy levels at a probability level of $P < 0.01$.

† Including Tifton 10, which was collected in Shanghai, China (Hanna et al., 1990).

further demonstrated that the genetic distances among the tetraploidy, pentaploidy, and hexaploidy are significant ($P < 0.01$). Within ploidy levels, the range of GSCs and the average GSC of tetraploids were smaller than those of the hexaploids and pentaploids, showing that the Chinese tetraploid *Cynodon* contained much wider genetic diversity compared with the pentaploid and hexaploid cytotypes.

## DISCUSSION

Among the 132 accessions used in this study, the 116 tetraploid *Cynodon* accessions (87.9%) originated from 11 provincial regions, clearly indicating this to be the predominant cytotype over the geographic expanse represented by the collection. In three western provinces, 96 of 101 accessions were tetraploids. In the other eight eastern and southern provincial regions, 20 of the 31 accessions were tetraploids. The wide distribution of the tetraploid cytotype in China is consistent with previous reports (Harlan and de Wet, 1969; Harlan et al., 1970a) that tetraploid *C. dactylon* var. *dactylon* is truly cosmopolitan. Although the tetraploid accessions were collected from two distinct geographic regions in China (Fig. 1), discrete genetic differentiation within a region is not observed and genetic overlap existed between the two separate regions indicated by AFLP markers in the dendrogram (Fig. 3) and principal coordinate plot (Fig. 4). This might be explained by the open pollination behavior of *Cynodon* plants. The predominant mode of sexual reproduction in *C. dactylon* is outcrossing due to cross-pollination and self-incompatibility (Burton, 1947, 1965; Taliaferro and Lamle, 1997a). Cross-pollination results in gene flow between natural populations, which probably prevents formation of distinctly differentiated genetic groups. We believe that genetic variation and differentiation patterns of the Chinese tetraploid *Cynodon* germplasm pool in the main distribution regions is of a gradual mode for tetraploid forms without apparent genetic barriers.

The pentaploid and hexaploid accessions are considered to belong to the taxon *C. dactylon* as described by Harlan and de Wet (1969) and Harlan et al. (1970a). The pentaploid and hexaploid accessions are included in *C. dactylon* based on similarity of morphological features compared with tetraploid accessions. The descriptions of *C. dactylon* var. *dactylon* by Harlan and de Wet (1969) and Harlan et al. (1970a) indicated the species contained only the tetraploid cytotype. Our AFLP marker data basically supported the morphological classification. A dendrogram from the cluster analysis (Fig. 3) showed that hexaploid and pentaploid accessions formed clusters or subclusters in mixture with or close to tetraploid accessions that originated in the same geographic region as the pentaploids and hexaploids. The spread of tetraploid, pentaploid, and hexaploid forms in a two-dimensional plot of principal coordinate analysis (Fig. 4), in which principal coordinates 1 (PC1) and 2 (PC2) accounted for 10.4% and 7.2% of total variation, respectively, is basically consistent with the dendrogram (Fig. 3). The close genetic relatedness of the three different cytotypes indicates they have a recent common ancestry. Hexaploidy and pentaploidy are rare in *Cynodon*. Powell et al. (1968) reported a hexaploid clone from a cross of tetraploid × diploid parents, and speculated that doubling of chromosome number at an early zygote stage probably accounted for its occurrence. Another hexaploid plant was identified in the progeny of a self-pollinated plant of tetraploid *C. dactylon* (Felder, 1967). Felder hypothesized that the autohexaploid plant resulted from the union of an unreduced female gamete and a reduced male gamete. Accordingly, the origin of the Chinese hexaploid forms most likely resulted from the union of reduced and unreduced gametes of tetraploid parents. The pentaploid forms probably originated from the union of one normal gamete from a tetraploid parent with another normal gamete from a hexaploid parent.

This is the first report of pentaploid *Cynodon* plants occurring naturally. The pentaploids were collected in subtropical environments, two from Hainan Island and the third from Fujian province. We are aware of only two previous reports of pentaploid *Cynodon* plants. Johnston (1975) found three pentaploid plants among progeny from a hexaploid plant (derived from interspecific hybridization of *C. barberi* and *C. dactylon*) crossed with a tetraploid *C. dactylon* plant. Burton et al. (1993) reported 'Tifton 85', an $F_1$ interspecific hybrid from a *C. dactylon* × *C. nlemfuensis* cross, to be a pentaploid.

Among the six triploids, five were morphologically similar to hybrids from *C. dactylon* var. *dactylon* by *C. transvaalensis* crosses because of short and slender racemes, two to four per inflorescence, fine leaves and stolons, low growing and very dense sod, while one (A12261) was close to *C. dactylon* var. *dactylon.* The five triploids were probably introduced plants based on their morphological and genetic similarity to Tifgreen and Tifway and their dissimilarity to the Chinese tetraploid accessions. Turf type triploid *Cynodon* cultivars developed in the USA have been introduced and widely used in China (Wu et al., 2001). The morphological traits of accessions 12256 and 12369 grown in greenhouse and field plots were very similar, but different from Tifgreen in leaf length and sod density (data not reported). Accessions 12256 and 12369 probably were cultivars introduced to China and may be mutational variants of Tifgreen or 'Tifdwarf'. Caetano-Anolles (1998b) reported the genetic instability of Tifgreen and Tifdwarf detected by DAF and ASAP (arbitrary signatures from amplification profiles) analysis. Off-types of Tifgreen were phenotypically distinct from the wild type in leaf texture, length, and color, stolon internode length, and other morphological characteristics, but genetically close to each other (Caetano-Anolles, 1998b). Accessions 12260, 12272, and 12282 were morphologically uniform, but distinct from Tifway in foliage color and developmental characteristics (data not shown), particularly in the late part of a growing season. Tifway tended to have darker green color and fewer inflorescences than the other three accessions in the fall (data not reported). Caetano-Anolles et al. (1997) indicated

that Tifway was genetically stable; thus, it is less likely that the three Chinese accessions are mutational variants of Tifway.

## CONCLUSIONS

Tetraploid *C. dactylon* is prevalent among *Cynodon* germplasm indigenous to the 11 provinces of China represented in the collection studied. Southeast China may represent a unique geographic area relative to the frequency of occurrence of hexaploid and pentaploid *Cynodon dactylon* cytotypes. Genetic differentiation among the three cytotypes is distinct based on AFLP markers. Though a more comprehensive collection is needed to elucidate genetic variation within *Cynodon* indigenous to China, this study demonstrates that substantial genetic variation exists within Chinese *C. dactylon* germplasm that might contribute to genetic improvement of the species.

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