Ploidy Level and DNA Content of Perennial Ryegrass Germplasm as Determined by Flow Cytometry

Ying Wang, Cale A. Bigelow, and Yiwei Jiang¹

Department of Agronomy, Purdue University, 915 W. State Street, West Lafayette, IN 47907-2054

Additional index words. *Lolium perenne* L., accession, diploid, tetraploid

Abstract. Perennial ryegrass (*Lolium perenne* L.) is a widely used cool-season turfgrass species. The exact ploidy levels of the worldwide perennial ryegrass accessions in the USDA National Plant Germplasm System (NPGS) are unknown, which could complicate future use and breeding efforts. The objective of this study was to determine the ploidy level and DNA content of the 194 USDA NPGS perennial ryegrass accessions and six commercial cultivars (Brightstar SLT, Catalina II, Divine, Inspire, Manhattan 4, Silver Dollar) using flow cytometry. Among the 200 accessions, 194 diploids and six tetraploids were identified. Three tetraploids originated from Canada with the remaining from Ireland, Japan, and The Netherlands. The average DNA content was 5.60 pg/2C for the diploid and 11.45 pg/2C for the tetraploid. The 2C DNA content was positively correlated ($r = 0.23$, $P < 0.01$) with seedling plant height but not seedling leaf width. This ploidy data provide important information for future marker trait analysis and cultivar improvement.

Perennial ryegrass is a principal cool-season grass species in temperate climates. Originating in Europe, temperate Asia, and North Africa, it is a self-incompatible species and is widely used for forage and turf industries in the United States, Europe, Japan, Australia, and New Zealand (Thorogood, 2003). In the United States, turf-type perennial ryegrasses are commonly used for golf courses, sports fields, lawns, and landscapes.

Perennial ryegrass is naturally diploid; however, tetraploids have been developed to improve forage quality, productivity, and turfgrass management (Barker et al., 2001; Richardson et al., 2007). As breeders continue to improve the germplasm, the future use of perennial ryegrass could be complicated if the ploidy level of known germplasms is unclear. The mixed ploidy levels in the grass population could also be challenging to marker development and gene identification, ultimately influencing marker-assisted selection to improve desirable traits. In addition to ploidy effects on breeding, use of diploid or tetraploid of perennial ryegrass has a significant impact on turf management. Richardson et al. (2007) found that diploid perennial ryegrass had superior turf appearance than the tetraploid perennial ryegrass when used for winter overseeding in dormant bermudagrass (*Cynodon* spp.) in three southern U.S. environments: Maricopa, AZ (arid), Fayetteville, AR (transition zone), and Auburn, AL (humid, subtropical). By contrast, tetraploid perennial ryegrass and meadow fescue (*Festuca pratensis* Huds.) transitioned more eas-

ily back to bermudagrass. The low summer survival of tetraploid perennial ryegrass needs to be strongly considered when developing perennial ryegrass for more permanent turf situations.

The traditional method for determining ploidy level is to count the number of chromosomes in a single plant cell that has proven reliable and has been used for different species (Lackey, 1980; Weedin and Powell, 1978). However, counting chromosomes is a time- and labor-consuming process (Lackey, 1980), particularly when a large set of samples is examined. Because plants differing in ploidy level may vary in growth habits, attempts have been made to distinguish ploidy level through morphological characteristics. This method, however, has not proven always accurate or sufficient. Flow cytometry is the newest technique to determine ploidy level, which appears to become a powerful method. This technique combines the advantages of microscopy and biochemical analysis to measure quantitative traits such as DNA and RNA content and total cellular protein content with high precision and rapid throughput (Eaton et al., 2004; Muirhead et al., 1985).

Flow cytometry has been used to determine the ploidy level and DNA content in several turfgrass species (Barker et al., 2001; Bonos et al., 2002; Eaton et al., 2004; Goldman et al., 2004; Wu et al., 2006). Diploid, tetraploid, and hexaploidy were found in six *Agrostis* species (Bonos et al., 2002); and triploid, tetraploid, hexaploid, and pentaploid were identified among the Chinese *Cynodon* accessions (Wu et al., 2006). By using flow cytometry, Eaton et al. (2004) found that DNA contents of Kentucky bluegrass (*Poa pratensis* L.) genotypes from the 22 cultivars ranged from 5.39 to 17.69 pg of DNA/2C. The positive correlations between

nuclear DNA content and cell size, seed size, and single leaf size were observed in 15 cultivated populations of perennial ryegrass (Sugiyama et al., 2002). Barker et al. (2001) tested DNA content in three tetraploid cultivars each of perennial ryegrass, annual or Italian ryegrass (*Lolium multiflorum* L.), and intermediate ryegrass and concluded that flow cytometry easily determined ploidy level and was sensitive enough to detect aneuploid plants.

Although perennial ryegrass ploidy level and DNA content have been previously determined using flow cytometry, the existing data are limited to only several cultivars. The ploidy level and DNA content of globally collected perennial ryegrass germplasm accessions has not been determined. Ploidy information of perennial ryegrass accessions worldwide is important and necessary for establishing natural diploid plant populations for trait and gene association analysis to avoid any potential complications resulting from mixed ploidy levels. Plant populations with a uniform ploidy level would also be of great value for studying the genetics of perennial ryegrass. Therefore, the objective of this study was to determine the ploidy level and DNA content of the 194 USDA National Plant Germplasm System (NPGS) perennial ryegrass accessions and six commercial cultivars using flow cytometry.

Materials and Methods

Plant materials. The perennial ryegrasses used in this study included 194 accessions obtained from the USDA NPGS at the Western Regional Plant Introduction Station in Pullman, WA, and six commercial cultivars (BrightStar SLT, Catalina II, Divine, Inspire, Manhattan 4, and Silver Dollar) (Turf-Seed Company, Gervais, OR; Scotts Inc., Marysville, OH). These cultivars represented a range of turf quality and other characteristics from those evaluated in the National Turfgrass Evaluation Program perennial ryegrass field test (NTEP, 2003). The selection of perennial ryegrass materials was based on their geographical locations to maximize ecotypic diversity (Table 1). The accessions were seeded in pots (9 cm deep and 10 cm in diameter) containing a sandy loam soil with a pH of 6.9 in a greenhouse at Purdue University. The grasses were fertilized weekly with a soluble fertilizer (24N–8P–16K) (Scotts Inc., Marysville, OH) to provide 146 kg·ha⁻¹ nitrogen annually. Irrigation was applied as necessary to prevent wilting. Three weeks after germination, plant height and leaf width were recorded for assessing morphological characteristics at the early stage of growth.

Flow cytometry. DNA content per nucleus was determined according to the method of Arumuganathan and Earle (1991). An approximate 150 mg of fresh perennial ryegrass leaf tissue of each accession and 30 mg of sorghum (*Sorghum bicolor* L.) leaf tissue (served as an internal standard) (Johnston et al., 1999) were mixed and cut into 0.5- to 1.0-mm segments in a petri dish (on ice)

Received for publication 25 Aug. 2009. Accepted for publication 28 Sept. 2009.

¹To whom reprint requests should be addressed; e-mail yjiang@purdue.edu.

Table 1. Accession number (No.), origin, mean DNA contents (DNA), and ploidy level of 194 perennial ryegrass accessions from the USDA National Plant Germplasm system and six commercial cultivars.

No.	Origin	DNA $\pm$ SE (pg)	Ploidy	No.	Origin	DNA $\pm$ SE (pg)	Ploidy
220178	Afghanistan	5.29 $\pm$ 0.05	Diploid	403907	Canada	5.60 $\pm$ 0.13	Diploid
220528	Afghanistan	5.64 $\pm$ 0.08	Diploid	403899	Canada	5.65 $\pm$ 0.21	Diploid
220597	Afghanistan	5.28 $\pm$ 0.08	Diploid	265351	Chile	5.69 $\pm$ 0.13	Diploid
317452	Afghanistan	5.61 $\pm$ 0.11	Diploid	206376	Cyprus	5.02 $\pm$ 0.21	Diploid
231578	Algeria	5.72 $\pm$ 0.09	Diploid	204085	Cyprus	5.77 $\pm$ 0.20	Diploid
231588	Algeria	5.80 $\pm$ 0.22	Diploid	277848	Cyprus	5.59 $\pm$ 0.19	Diploid
231576	Algeria	5.53 $\pm$ 0.29	Diploid	182857	Czech Republic	5.25 $\pm$ 0.09	Diploid
231580	Algeria	5.52 $\pm$ 0.26	Diploid	321397	Czech Republic	5.76 $\pm$ 0.09	Diploid
231587	Algeria	5.62 $\pm$ 0.22	Diploid	200322	Denmark	5.43 $\pm$ 0.25	Diploid
231589	Algeria	5.96 $\pm$ 0.41	Diploid	225825	Denmark	5.52 $\pm$ 0.16	Diploid
231594	Algeria	5.53 $\pm$ 0.25	Diploid	303027	Denmark	5.69 $\pm$ 0.19	Diploid
202451	Argentina	5.32 $\pm$ 0.06	Diploid	303028	Denmark	5.52 $\pm$ 0.21	Diploid
275660	Australia	5.52 $\pm$ 0.15	Diploid	239730	Egypt	4.94 $\pm$ 0.44	Diploid
284823	Australia	5.49 $\pm$ 0.23	Diploid	197270	Finland	5.44 $\pm$ 0.19	Diploid
284826	Australia	5.66 $\pm$ 0.03	Diploid	268333	Former Soviet Union	5.30 $\pm$ 0.11	Diploid
285101	Australia	5.87 $\pm$ 0.19	Diploid	440474	Former Soviet Union	5.54 $\pm$ 0.20	Diploid
187222	Belgium	5.52 $\pm$ 0.24	Diploid	505840	Former Soviet Union	5.86 $\pm$ 0.17	Diploid
303044	Belgium	5.51 $\pm$ 0.27	Diploid	505841	Former Soviet Union	5.72 $\pm$ 0.16	Diploid
418721	Belgium	5.65 $\pm$ 0.45	Diploid	505842	Former Soviet Union	5.56 $\pm$ 0.08	Diploid
598437	Belgium	5.54 $\pm$ 0.13	Diploid	505843	Former Soviet Union	5.90 $\pm$ 0.10	Diploid
306292	Bolivia	5.31 $\pm$ 0.16	Diploid	418726	France	5.54 $\pm$ 0.25	Diploid
371952	Bulgaria	5.66 $\pm$ 0.14	Diploid	418727	France	5.86 $\pm$ 0.19	Diploid
628717	Bulgaria	5.67 $\pm$ 0.10	Diploid	265340	France	5.39 $\pm$ 0.20	Diploid
403877	Canada	12.00 $\pm$ 0.50	Tetraploid	265341	France	5.56 $\pm$ 0.08	Diploid
403889	Canada	5.50 $\pm$ 0.33	Diploid	303026	France	5.69 $\pm$ 0.14	Diploid
403903	Canada	11.79 $\pm$ 0.29	Tetraploid	321681	France	5.73 $\pm$ 0.03	Diploid
278773	Canada	6.11 $\pm$ 0.12	Diploid	418741	France	5.79 $\pm$ 0.09	Diploid
403846	Canada	11.56 $\pm$ 0.14	Tetraploid	234779	Germany	5.69 $\pm$ 0.24	Diploid
403847	Canada	5.71 $\pm$ 0.18	Diploid	231597	Greece	6.01 $\pm$ 0.08	Diploid
403851	Canada	5.69 $\pm$ 0.15	Diploid	231598	Greece	6.31 $\pm$ 0.38	Diploid
403868	Canada	5.46 $\pm$ 0.21	Diploid	241608	Greece	5.40 $\pm$ 0.14	Diploid
403869	Canada	5.73 $\pm$ 0.15	Diploid	504539	Greece	5.40 $\pm$ 0.49	Diploid
403886	Canada	5.66 $\pm$ 0.19	Diploid	298091	Hungary	5.82 $\pm$ 0.17	Diploid
290368	Hungary	5.77 $\pm$ 0.24	Diploid	231595	Morocco	6.17 $\pm$ 0.18	Diploid
290373	Hungary	5.70 $\pm$ 0.28	Diploid	516605	Morocco	5.30 $\pm$ 0.06	Diploid
632542	Hungary	5.68 $\pm$ 0.25	Diploid	598839	Morocco	5.24 $\pm$ 0.14	Diploid
229702	Iran	5.68 $\pm$ 0.17	Diploid	598869	Morocco	5.31 $\pm$ 0.03	Diploid
223385	Iran	5.37 $\pm$ 0.28	Diploid	598877	Morocco	5.24 $\pm$ 0.09	Diploid
227020	Iran	5.25 $\pm$ 0.08	Diploid	598892	Morocco	5.28 $\pm$ 0.06	Diploid
231619	Iran	5.55 $\pm$ 0.24	Diploid	303022	Netherlands	5.64 $\pm$ 0.11	Diploid
231620	Iran	5.60 $\pm$ 0.23	Diploid	303031	Netherlands	5.79 $\pm$ 0.15	Diploid
547390	Iran	5.40 $\pm$ 0.19	Diploid	237184	Netherlands	5.36 $\pm$ 0.21	Diploid
254898	Iraq	5.70 $\pm$ 0.29	Diploid	237187	Netherlands	5.58 $\pm$ 0.11	Diploid
265344	Ireland	5.56 $\pm$ 0.07	Diploid	266293	Netherlands	5.75 $\pm$ 0.13	Diploid
265342	Ireland	5.61 $\pm$ 0.23	Diploid	303021	Netherlands	5.84 $\pm$ 0.17	Diploid
265349	Ireland	5.91 $\pm$ 0.35	Diploid	303048	Netherlands	11.37 $\pm$ 0.64	Tetraploid
578768	Ireland	10.89 $\pm$ 0.86	Tetraploid	462339	New Zealand	5.66 $\pm$ 0.14	Diploid
418714	Italy	5.73 $\pm$ 0.22	Diploid	189392	New Zealand	5.63 $\pm$ 0.18	Diploid
418706	Italy	5.88 $\pm$ 0.18	Diploid	238938	New Zealand	5.73 $\pm$ 0.15	Diploid
418712	Italy	5.64 $\pm$ 0.17	Diploid	376878	New Zealand	5.91 $\pm$ 0.32	Diploid
598434	Italy	5.58 $\pm$ 0.25	Diploid	462335	New Zealand	5.66 $\pm$ 0.05	Diploid
598928	Italy	5.35 $\pm$ 0.14	Diploid	462336	New Zealand	5.73 $\pm$ 0.13	Diploid
610928	Italy	5.51 $\pm$ 0.13	Diploid	462337	New Zealand	5.65 $\pm$ 0.34	Diploid
610965	Italy	5.92 $\pm$ 0.06	Diploid	577269	Norway	5.83 $\pm$ 0.12	Diploid
636539	Italy	5.60 $\pm$ 0.27	Diploid	610802	Norway	5.52 $\pm$ 0.34	Diploid
420124	Japan	5.37 $\pm$ 0.06	Diploid	619003	Norway	5.57 $\pm$ 0.13	Diploid
420126	Japan	5.80 $\pm$ 0.15	Diploid	267059	Poland	5.85 $\pm$ 0.10	Diploid
420127	Japan	11.50 $\pm$ 0.95	Tetraploid	267058	Poland	5.82 $\pm$ 0.12	Diploid
420128	Japan	5.88 $\pm$ 0.25	Diploid	272120	Poland	5.49 $\pm$ 0.25	Diploid
636643	Japan	5.44 $\pm$ 0.11	Diploid	274637	Poland	5.71 $\pm$ 0.28	Diploid
231569	Libya	5.52 $\pm$ 0.11	Diploid	384478	Poland	5.49 $\pm$ 0.26	Diploid
231565	Libya	5.31 $\pm$ 0.10	Diploid	231605	Portugal	5.65 $\pm$ 0.09	Diploid
231566	Libya	5.65 $\pm$ 0.13	Diploid	231604	Portugal	5.65 $\pm$ 0.17	Diploid
231567	Libya	5.57 $\pm$ 0.09	Diploid	231606	Portugal	5.67 $\pm$ 0.24	Diploid
231568	Libya	5.43 $\pm$ 0.25	Diploid	418707	Romania	5.61 $\pm$ 0.09	Diploid
418722	Luxembourg	5.30 $\pm$ 0.14	Diploid	311075	Romania	5.48 $\pm$ 0.15	Diploid
418723	Luxembourg	5.56 $\pm$ 0.08	Diploid	418708	Romania	5.84 $\pm$ 0.14	Diploid
577254	Luxembourg	5.50 $\pm$ 0.20	Diploid	598453	Romania	5.66 $\pm$ 0.16	Diploid
619474	Romania	5.07 $\pm$ 0.30	Diploid	303032	UK	5.77 $\pm$ 0.18	Diploid
502412	Russian Federation	5.49 $\pm$ 0.30	Diploid	577260	UK	5.65 $\pm$ 0.34	Diploid
538976	Russian Federation	5.51 $\pm$ 0.08	Diploid	577265	UK	5.57 $\pm$ 0.13	Diploid

(Continued on next page)

Table 1. (Continued) Accession number (No.), origin, mean DNA contents (DNA), and ploidy level of 194 perennial ryegrass accessions from the USDA National Plant Germplasm system and six commercial cultivars.

No.	Origin	DNA $\pm$ SE (pg)	Ploidy	No.	Origin	DNA $\pm$ SE (pg)	Ploidy
611036	Russian Federation	5.80 $\pm$ 0.18	Diploid	577268	UK	5.61 $\pm$ 0.12	Diploid
410155	South Africa	5.40 $\pm$ 0.09	Diploid	595046	UK	5.76 $\pm$ 0.23	Diploid
287855	Spain	5.72 $\pm$ 0.13	Diploid	598447	UK	5.41 $\pm$ 0.11	Diploid
423136	Spain	5.41 $\pm$ 0.25	Diploid	598452	UK	5.85 $\pm$ 0.07	Diploid
287849	Spain	5.10 $\pm$ 0.30	Diploid	610815	UK	5.69 $\pm$ 0.41	Diploid
287852	Spain	5.13 $\pm$ 0.20	Diploid	610818	UK	5.36 $\pm$ 0.28	Diploid
319018	Spain	5.72 $\pm$ 0.19	Diploid	619012	UK	5.62 $\pm$ 0.16	Diploid
198070	Sweden	5.41 $\pm$ 0.07	Diploid	619554	UK	5.78 $\pm$ 0.13	Diploid
265336	Sweden	5.67 $\pm$ 0.17	Diploid	Inspire	USA	5.58 $\pm$ 0.12	Diploid
303020	Sweden	5.66 $\pm$ 0.23	Diploid	Silver Dollar	USA	5.46 $\pm$ 0.18	Diploid
303037	Sweden	6.01 $\pm$ 0.13	Diploid	Brightstar SLT	USA	5.75 $\pm$ 0.09	Diploid
418736	Switzerland	5.43 $\pm$ 0.27	Diploid	Manhattan 4	USA	5.53 $\pm$ 0.20	Diploid
598441	Switzerland	5.57 $\pm$ 0.16	Diploid	578760	USA	5.75 $\pm$ 0.14	Diploid
598443	Switzerland	5.64 $\pm$ 0.13	Diploid	578763	USA	5.75 $\pm$ 0.12	Diploid
598911	Tunisia	5.59 $\pm$ 0.23	Diploid	578769	USA	5.32 $\pm$ 0.08	Diploid
610925	Tunisia	5.54 $\pm$ 0.24	Diploid	Divine	USA	5.57 $\pm$ 0.17	Diploid
610926	Tunisia	5.52 $\pm$ 0.19	Diploid	Catalina II	USA	5.57 $\pm$ 0.15	Diploid
610950	Tunisia	5.36 $\pm$ 0.30	Diploid	600783	USA	5.89 $\pm$ 0.24	Diploid
204879	Turkey	5.76 $\pm$ 0.16	Diploid	600878	USA	5.69 $\pm$ 0.05	Diploid
204881	Turkey	5.34 $\pm$ 0.19	Diploid	632517	USA	5.54 $\pm$ 0.07	Diploid
204880	Turkey	5.80 $\pm$ 0.17	Diploid	634204	USA	5.13 $\pm$ 0.29	Diploid
340104	Turkey	5.30 $\pm$ 0.07	Diploid	634205	USA	5.45 $\pm$ 0.07	Diploid
340108	Turkey	5.61 $\pm$ 0.09	Diploid	634278	USA	5.47 $\pm$ 0.07	Diploid
577273	Turkey	5.53 $\pm$ 0.12	Diploid	577796	USA	5.87 $\pm$ 0.14	Diploid
598515	Turkey	5.74 $\pm$ 0.26	Diploid	251141	Yugoslavia	6.12 $\pm$ 0.39	Diploid
598517	Turkey	5.45 $\pm$ 0.06	Diploid	251224	Yugoslavia	5.62 $\pm$ 0.25	Diploid
598518	Turkey	5.60 $\pm$ 0.06	Diploid	251667	Yugoslavia	5.59 $\pm$ 0.08	Diploid
303011	UK	5.58 $\pm$ 0.13	Diploid	277846	Yugoslavia	5.43 $\pm$ 0.31	Diploid
303012	UK	5.80 $\pm$ 0.10	Diploid	577251	Yugoslavia	5.68 $\pm$ 0.19	Diploid

containing 1.5 mL of solution A [10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mM KCL, 5 mM HEPES, 1.0 mg/mL dithiothreitol, 30 μL propidium iodide stock (5 mg/mL), and 37.5 μL Triton X-100 stock (10% w/v), adjusted to pH 7.0]. The excised leaf tissue was then filtered through 30- μm nylon mesh into a microcentrifuge tube and centrifuged at $12,000 \times g$ for 30 s. The supernatant was discarded and the pellet was resuspended in 500 μL of solution B [7.5 mL solution A; 17.5 μL RNase (DNase-free)]. The resuspension was incubated in a 37 °C water bath for 15 min and analyzed immediately using a Cytomics FC 500 flow cytometer (Beckman Coulter, Miami, FL) with a 488-nm air-cooled argon laser and the 633 HeNe. For each sample, $\approx 5,000$ to 10,000 nuclei were collected and analyzed by the Cytomics Expo32 software. The amount of propidium iodide (PI) intercalating in the double-stranded DNA is proportional to DNA content for the sample. The fluorescence intensity of PI measured by the flow cytometer is also proportional to DNA content per cell.

Flow cytometer records fluorescence intensity of cells in G1 and G2 periods for both the internal standard and the plant of interest. Cells in the G1 period precede DNA synthesis (S). Cells in the G2 period have finished DNA replication but have not split. Thus, 2C DNA content can be calculated based on the value of fluorescence intensity of G1 peaks for both the internal standard and the plant of interest. The DNA content of the samples was determined by the equation: nuclear DNA content = (mean position of sample peak)/(mean position of standard peak) $\times$ DNA content of the standard. Sorghum was chosen as the internal DNA standard based on the rule

that the G1 peak of an internal standard should be close to those of the target species but not overlap with each other. Consistent with a previous study examining the DNA content in perennial ryegrass (Barker et al., 2001), sorghum was selected from the list of plants that are recommended as excellent DNA content standards (Johnston et al., 1999).

Morphological measurements. Three weeks after seed germination, three individual plants (from three pots) for each accession were selected for measuring plant height and leaf width to correlate morphology with DNA content. Plant height was measured from the soil surface to the top of the uppermost leaf blade. Leaf width was measured at the widest point of the most mature leaf.

Experimental design and data analyses. The experiment was conducted as a randomized complete block design with three replications (three pots). A total of 200 perennial ryegrass accessions were assigned randomly within each block. Three replications were made for each accession to measure plant height and leaf width and conduct flow cytometry analysis. The mean plant height, leaf width, and 2C DNA content of three samplings for each diploid and tetraploid accession were used to conduct correlation analysis. Correlations between DNA content and morphological traits of 200 accessions were accomplished using Statistical Analysis System (SAS 9.1; SAS Institute, 2004).

Results and Discussion

The differences in DNA content between the diploid and tetraploids were indicated by the relative fluorescence intensity (Fig. 1).

Most of the cells were in the G1 period, except for meristem that contains actively dividing cells. Cells in the G2 period had double the amount of DNA as those in G1. Among 200 accessions, 194 diploids and six tetraploids were identified (Table 1), indicating that most of perennial ryegrasses are diploid in nature. The mean DNA content of the diploid and the tetraploids was 5.60 and 11.45 pg, respectively. The results were very close to those reported by Barker et al. (2001) in that the DNA content of diploid and tetraploid perennial ryegrass was 5.65 and 11.41 pg, respectively. The use of different internal standard and sampling procedures might cause the slight differences between individual studies. The DNA content of tetraploids was not exactly twofold to that of diploid. Bonos et al. (2002) also found similar results in six *Agrostis* species and they proposed that genome differences among species could be the reason for the DNA content of tetraploid to not be exactly double that of diploid. It is estimated that perennial ryegrass has a genome size of 5.66 pg/2C (Arumuganathan et al., 1999), and the repetitive DNA content is expected to be in the order of 75%. The deletion of repetitive DNA in the higher ploidy level could also result in the inconsistency of DNA contents (Bonos et al., 2002).

Among the 194 diploids, the DNA content ranged from 5.07 to 6.31 pg for grasses from Europe, 5.02 to 5.88 pg for grasses from Asia, 5.13 to 6.11 pg for grasses from North America, 5.31 to 5.69 pg for grasses from South America, 4.94 to 6.17 pg for grasses from Africa, and 5.49 to 5.91 pg for grasses from Oceania (Table 1). The grasses from

Fig. 1. Histogram of fluorescence intensity of propidium iodide (PI) from single cells in (A) diploid and (B) tetraploid accessions of perennial ryegrass compared with sorghum (internal standard). STD represents cells of the sorghum in G1 period. G0-G1 represents cells of perennial ryegrass in the G1 period. G2M represents cells of perennial ryegrass in the G2 period when cells contain double the amount of DNA content than those in the G1 period. The x axis is the fluorescence intensity of PI from a single cell. The y axis indicates the number of cells collected by flow cytometer.

Table 2. Plant height (ht) and leaf width at seedling stage and their correlations with 2C DNA content in perennial ryegrass.

	Plant ht (cm)	Leaf width (mm)
Diploid	15.6 ± 0.21	3.15 ± 0.05
Tetraploid	20.5 ± 1.07	3.72 ± 0.17
2C DNA content	0.23**z	0.08 NS

^zCorrelation was analyzed by using 200 accessions (mean of three samplings for each accession).

** and NS indicate significant at the $P < 0.01$ and nonsignificant, respectively.

Africa and Europe showed the largest variation in DNA content, whereas grasses from South America and Oceania had the smallest variation. Because 89% of the accessions used in this study came from Africa and Europe, the large sample size might contribute to the relatively large variation in DNA content of those samples. Six tetraploids were identified with DNA content ranging from 11.00 to 12.11 pg (Table 1). Three of them were from Canada and the other three were from The Netherlands, Japan, and Ireland, respectively. Two of three tetraploids from Canada (PI 403877 and 403903) were found in the perennial ryegrass core collections. Although only 3% of tetraploids were found in the selected accessions, they could complicate research results when choosing and using natural populations.

The average plant height and leaf width for diploid perennial ryegrass seedlings were

15.6 cm and 3.2 mm, respectively, whereas tetraploids had an average of 20.5 cm for plant height and 3.7 mm for leaf width (Table 2). Plant height was positively correlated with DNA content, but not with leaf width at the seedling stage (Table 2). Although the mean values for plant height and leaf width for tetraploids were greater than those of diploid, some diploids had similar height and leaf width to tetraploids (data not shown). Bonos et al. (2002) reported that flag leaf length was the only morphological measurement significantly positively correlated to DNA in six *Agrostis* species, and no other morphological measurements were associated with DNA content. These observations indicate that identification of ploidy level merely through morphological measurements is not a reliably accurate method.

In summary, the ploidy level of 200 worldwide perennial ryegrass accessions was successfully identified using flow cytometry. Among them, 194 diploids and six tetraploids were identified. The average DNA content was 5.60 pg/2C for the diploid and 11.45 pg/2C for the tetraploid. Determining ploidy level in perennial ryegrass helps establish natural diploid mapping populations to analyze genetic diversity and population structure necessary for marker trait association analysis. In addition, the six tetraploid accessions may also be potential resources for breeders to develop tetraploid perennial ryegrass cultivars for uses on winter-overseeding bermudagrass.

Literature Cited

- Arumuganathan, K. and E.D. Earle. 1991. Estimation of nuclear DNA content of plants by flow cytometry. *Plant Mol. Biol. Rpt.* 9:229-241.
- Arumuganathan, K., S.P. Tallury, M.L. Fraser, A.H. Bruneau, and R. Qu. 1999. Nuclear DNA content of thirteen turfgrass species by flow cytometry. *Crop Sci.* 39:1518-1521.
- Barker, R.E., J.A. Kilgore, R.L. Cook, A.E. Garay, and S.E. Warnke. 2001. Use of flow cytometry to determine ploidy level of ryegrass. *Seed Sci. Tech.* 29:493-502.
- Bonos, S.A., K.A. Plumley, and W.A. Meyer. 2002. Ploidy determination in *Agrostis* using flow cytometry and morphological traits. *Crop Sci.* 42:192-196.
- Eaton, T.D., J. Curley, R.C. Williamson, and G. Jung. 2004. Determination of the level of variation in polyploidy among Kentucky bluegrass cultivars by means of flow cytometry. *Crop Sci.* 44:2168-2174.
- Goldman, J.J., W.W. Hanna, and G.H. Fleming. 2004. Ploidy variation among herbicide-resistant bermudagrass plants of cv. TifEagle transformed with the *bar* gene. *Plant Cell Rpt.* 22:553-560.
- Johnston, J.S., M.D. Bennett, A.L. Rayburn, D.W. Galbraith, and H.J. Price. 1999. Reference standards for determination of DNA content of plant nuclei. *Amer. J. Bot.* 86:609-613.
- Lackey, J.A. 1980. Chromosome numbers in the Phaseoleae (Fabaceae: Faboideae) and their relation to taxonomy. *Amer. J. Bot.* 67:595-602.
- Muirhead, K.A., P.K. Horan, and G. Poste. 1985. Flow cytometry: Present and future. *Bio/technology* 3:337-356.
- NTEP. 2003. National Turfgrass Evaluation Program. <<http://www.ntep.org>>.
- Richardson, M.D., K.W. Hignight, R.H. Walker, C.A. Rodgers, D. Rush, J.H. McCalla, and D.E. Karcher. 2007. Meadow fescue and tetraploid perennial ryegrass-two new species for overseeding dormant bermudagrass turf. *Crop Sci.* 47:83-90.
- SAS Institute. 2004. SAS user's guide: Statistics. SAS Inst., Cary, NC.
- Sugiyama, S., K. Yamaguchi, and T. Yamada. 2002. Intraspecific phenotypic variation associated with nuclear DNA content in *Lolium perenne* L. *Euphytica* 128:145-151.
- Thorogood, D. 2003. Perennial ryegrass (*Lolium perenne* L.), p. 75-105. In: Casler, M.D. and R.R. Duncan (eds.). *Turfgrass biology, genetics and breeding*. John Wiley & Sons, Inc. Hoboken, NJ.
- Weedin, J.F. and A.M. Powell. 1978. Chromosome numbers in Chihuahuan Desert Cactaceae. *Trans-Pecos Texas. Amer. J. Bot.* 65: 531-537.
- Wu, Y.Q., C.M. Taliaferro, G.H. Bai, D.L. Martin, J.A. Anderson, M.P. Anderson, and R.M. Edwards. 2006. Genetic analyses of Chinese *Cynodon* accessions by flow cytometry and AFLP markers. *Crop Sci.* 46:917-926.