Running title: *Festuca ovina* USDA PI DNA ploidy estimation

Title: **DNA Content and Ploidy Estimation of *Festuca ovina* Accessions by Flow Cytometry**

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Abbreviations: USDA, United States Department of Agriculture; NPGS, National Plant Germplasm System;

ABSTRACT: *Festuca ovina* is a fine fescue that is used as a low-input turfgrass. The ploidy levels of *F. ovina* accessions held by the USDA National Plant Germplasm System (NPGS) are unknown, limiting the use of the germplasm in breeding programs. The objective of this study was to determine DNA content and estimate ploidy of these 127 accessions. Among the accessions, we identified a wide range of ploidy levels from diploid to octoploid. We also found the accessions with higher ploidy levels usually had larger seed size. These results will be informative to plant breeders and researchers using germplasm from the *F. ovina* collection and point to challenges in maintaining polyploid, outcrossing germplasm seed stocks in common nurseries.

INTRODUCTION

Fine fescues (*Festuca* spp. L.) are a diverse group of grasses characterized by fine leaf texture. Fine fescues are native to Eurasia but have been introduced and naturalized to many temperate regions in the world (Barkworth, Capels, Long, Anderton, & Piep, 2007; Beard, 1972; Vasey, 1883). Theses grasses are used for forage, ornamental purposes, and particularly as low-input turfgrasses. The group comprises genetically diverse taxa, including hard fescue (*Festuca brevipila* Tracey, 2n=6x=42), sheep fescue (*F. ovina* L., 2n=4x=28), strong creeping red fescue (*Festuca rubra* ssp. *rubra* 2n=8x=56), slender creeping red fescue [*Festuca rubra* ssp. *litoralis* (G. Mey.) Auquier 2n=6x=42], and Chewings fescue [*Festuca rubra* ssp. *fallax* (Thuill.) Nyman 2n=6x=42] ((Ruemmele, Brilman, & Huff, 1995).

Based on their morphology, cytology, and chloroplast-genome-based phylogenetic relationship, fine fescues are divided in two complexes: the *F. ovina* complex, which includes *F. brevipila* and *F. ovina*, and the *F. rubra* complex, which includes *F. rubra* ssp. *litoralis*, *F. rubra* ssp. *rubra*, and *F. rubra* ssp. *fallax* (Huff & Palazzo, 1998; Qiu, Hirsch, Yang, & Watkins, 2019; Wilkinson & Stace, 1991). Species in *F. ovina* are bunch-type and non-rhizomatous while subspecies in the *F. rubra* complex can be rhizomatous (ssp. *litoralis* and ssp. *rubra*) and bunch-type (ssp. *fallax*). Taxon identification within the *F. ovina* complex is difficult because of morphological and ecotype diversity (Piper, 1906; Schmit, Duell, & Funk, 1974). Previously, leaf color has been used for species identification; however, in the United States, sheep fescue is described as having a bluish-gray leaf color and hard fescue leaf blade color is considered green (Beard, 1972), while in Europe, it is the opposite (Hubbard, 1968). The *F. ovina* complex also includes some other taxa beyond those commonly used as turfgrasses that have ploidy level varying from diploid (2n=2x=14) such as *Festuca ovina* ssp. *ovina*, tetraploid *Festuca*

armoricana, and hexaploid *Festuca huonii* (Seal, 1983; Stace, 2010; Wilkinson & Stace, 1991). All of these factors make the identification of *F. ovina* challenging.

Turfgrass breeding and genetics objectives are focused on aesthetic beauty, disease resistance, drought tolerance, and traits associated with reduced inputs (Bonos, Clarke, & Meyer, 2006; Bonos & Huff, 2013; Casler, 2003; Clarke et al., 2006). Plant breeders seek desirable alleles in exotic accessions and attempt to introgress them into the existing cultivars. One of the most valuable resources for breeders is the USDA-ARS National Plant Germplasm System (NPGS), which holds more than 500,000 accessions that represent more than 10,000 plant species. These accessions have been widely used in plant breeding programs for abiotic and biotic stress improvement (Chang & Hartman, 2017; Christensen et al., 2007; Dilday, Lin, & Yan, 1994; Leng, Wang, Ali, Zhao, & Zhong, 2016; Nelson, Amdor, & Orf, 1987). To improve turfgrass cultivars by utilizing the germplasm accessions, it is important to know the ploidy level of the accessions used to avoid hybridizing plants with different ploidy levels that results in nonviable offspring. Additionally, without knowing the ploidy level, genetics and phenotyping of these germplasm could lead to incorrect interpretation of results.

Traditional plant breeding methods that emphasize hybridizing elite germplasm usually result in the loss of genetic diversity and heterosis which could result in greater susceptibility to important stresses (Christiansen, Andersen, & Ortiz, 2002; Melchinger, 1999; Reif et al., 2005). The use of exotic germplasm has been a common practice in maintaining plant genetic diversity in the breeding process to reduce these problems and avoid breeding bottlenecks (Goodman, 1999; Mikel, Diers, Nelson, & Smith, 2010; Prasanna, 2012; Van Esbroeck & Bowman, 1998). In both cool-season and warm-season turfgrasses, genetic diversity of available germplasm has

been studied using either molecular markers or sequencing arrays (Baird et al., 2012; Budak, Shearman, Gaussoin, & Dweikat, 2004; Chen, Wang, Waltz, & Raymer, 2009).

For NPGS accessions from the *F. ovina* complex, it is necessary to determine ploidy level before conducting germplasm selection and performing hybridization; this has traditionally been done by counting chromosomes (Maluszynska, 2003; Vargas, McAllister, Morton, Jury, & Wilkinson, 1999), a reliable but time-consuming method which is made all the more challenging when dealing with the numerous and small (even under magnification) chromosomes of the fine fescue species. Flow cytometry is a powerful tool for DNA content measurement and allows researchers to estimate the ploidy level by comparing to known standards. This method is less time consuming, cheaper, and proven to work in grasses where it has been used to calculate the DNA content and estimate ploidy levels in Texas bluegrass (*Poa arachnifera* Torr.), buffalograss [*Bouteloua dactyloides* (Nutt.) Engelm.], perennial ryegrass (*Lolium perenne* L.) and fine fescues (Ka Arumuganathan & Earle, 1991; Goldman, 2015; Huff & Palazzo, 1998; Johnson, Kenworthy, Auld, & Riordan, 2001; Johnson, Riordan, & Arumuganathan, 1998; Qiu et al., 2019).

We used flow cytometry to determine the DNA content and ploidy level of 127 USDA *F. ovina* PI collections. In addition, we used image analysis to measure and compare the seed size on selected PI accessions among ploidy levels.

85 **MATERIAL AND METHODS**

86 **Plant Material**

87 A total of 127 accessions labeled as *Festuca ovina* from 20 countries were obtained from
88 the USDA Germplasm Resources Information Network (GRIN) in 2016 (**Table S1**).

89 Seeds of each accession were sown into greenhouse pots (four-inch size) filled with BRK
90 Promix soil (Premier Tech, USA) at the Plant Growth Facility at the University of Minnesota in
91 St. Paul. After reaching four to five leaf stage, five seedlings per accession were randomly
92 selected and transplanted into individual one-inch size cone container. Plants were grown with
93 16 h day and 8 hr night with bi-daily watering and weekly fertilization using Peat-lite 20-10-20
94 fertilizer (J.R. Peters Inc.) with supplemental ammonium sulfate and Sprint 330 (BASF, USA).
95 The five genotypes of each accession were vegetatively cloned into six replications of each
96 genotype and transplanted to a field nursery in at the Minnesota Agricultural Experiment Station
97 in St. Paul, MN. *F. ovina* cv. Quatro and *F. brevipila* cv. Beacon were also planted in the field as
98 standards.

99 **Flow Cytometry Procedure**

100 To determine the nucleus DNA content of accessions, flow cytometry was carried out
101 using the method described by Arumuganathan and Earle (1991). Because seeds used in this
102 study resulted from open pollination, we evaluated three genotypes for each accession to obtain a
103 more accurate ploidy representation of the population. When the three selected genotypes were
104 not at the same ploidy level, a fourth genotype was evaluated. The ploidy level of the accession
105 was determined by the ploidy of the majority genotypes (75%).

Fresh mature *F. ovina* leaf samples in the nursery field were harvested between 9-11 a.m. and trimmed to 1-2 cm and used for flow cytometry. Perennial ryegrass leaf tissue was harvested at the same time in the greenhouse. For each sample the flow cytometry staining solution contained 4.29 µL propidium iodide, 0.71 mL of CyStain UV Precise P staining buffer, and 2.14 µL RNAseA. To prepare plant tissue, a 0.5 cm x 0.5 cm leaf samples was excised into small pieces using a razor blade in 500 µL CyStain UV Precise P extraction buffer (Sysmex) and passed through a 50-µm size filter (Sysmex). The staining solution was added to the flow-through to stain the nuclei in each sample. Samples were stored on ice before loading the flow cytometer. Flow cytometry was carried out using the BD LSRII H4760 (LSRII) instrument (BD Biosciences, USA) with PI laser detector using 480V with a minimum of 1,000 events at the University of Minnesota Flow Cytometry Resource (UCRF). Data were visualized and analyzed on BD FACSDiva 8.0.1 software.

DNA Content Estimation and Ploidy Level Determination

Species DNA content was estimated following the method described by Dolezel and Bartoš (2005). The perennial ryegrass (*Lolium perenne*) 2C DNA content (2C = 5.66 pg/2C) served as the diploid DNA content standard (Arumuganathan, Tallury, Fraser, Bruneau, & Qu, 1999). Sample 2C DNA content was calculated using **Equation 1**. To estimate the ploidy level in *F. ovina* complex, diploid *F. ovina* PI 230246 measured in previous study was used (2C = 4.7 pg/2C); species ploidy level was estimated using **Equation 2**.

Equation 1

$$\text{Sample 2C DNA content} = \frac{\text{(sample G1 peak mean)}}{\text{(standard G1 peak mean)}} \times \text{standard 2C DNA content (pg DNA)}$$

Equation 2

$$\text{Sample ploidy} = \frac{\text{2n x sample pg/nucleus}}{\text{PI 230246 pg/nucleus}}$$

Seed Size Measurement and Comparison

For seed size measurements, we randomly picked 10 seeds from each of three accessions for each calculated ploidy level (a total of 12 accessions). Tetraploid *F. Ovina* cv. Quatro and hexaploid *F. brevipila* cv. Beacon were also included as references.

Seeds were spread on a digital scanner (Epson perfection v6 flatbed scanner, Nagano, Japan) and scanned at 1200 dots per inch (dpi) with the size of 2097 x 1624 pixels. The images were processed with a custom Matlab script (https://github.umn.edu/jbarreto/seed_morphology) that transformed the original images to measure the seed length and width respectively as the length (in pixels) of the major and minor axes of a fitted ellipse, whereas the area was calculated as the number of pixels in the seed. The seed area was calculated for each of 10 seeds for each of the 14 entries. The seed area was used to analyze the correlation between ploidy level and seed size and visualized using the ggplot2 package in R (Kahle & Wickham, 2013).

RESULTS

DNA Content Measurement and Ploidy Estimation

Festuca ovina accessions had a high level of DNA content variation with the smallest DNA content of 3.77 pg (2C) from PI 115358, and the largest genome 19.66 pg (2C) from PI 302899 (**Table S2**). Flow cytometry revealed the 127 accessions represented a range of ploidy levels from diploid to octoploid (**Figure 1**).

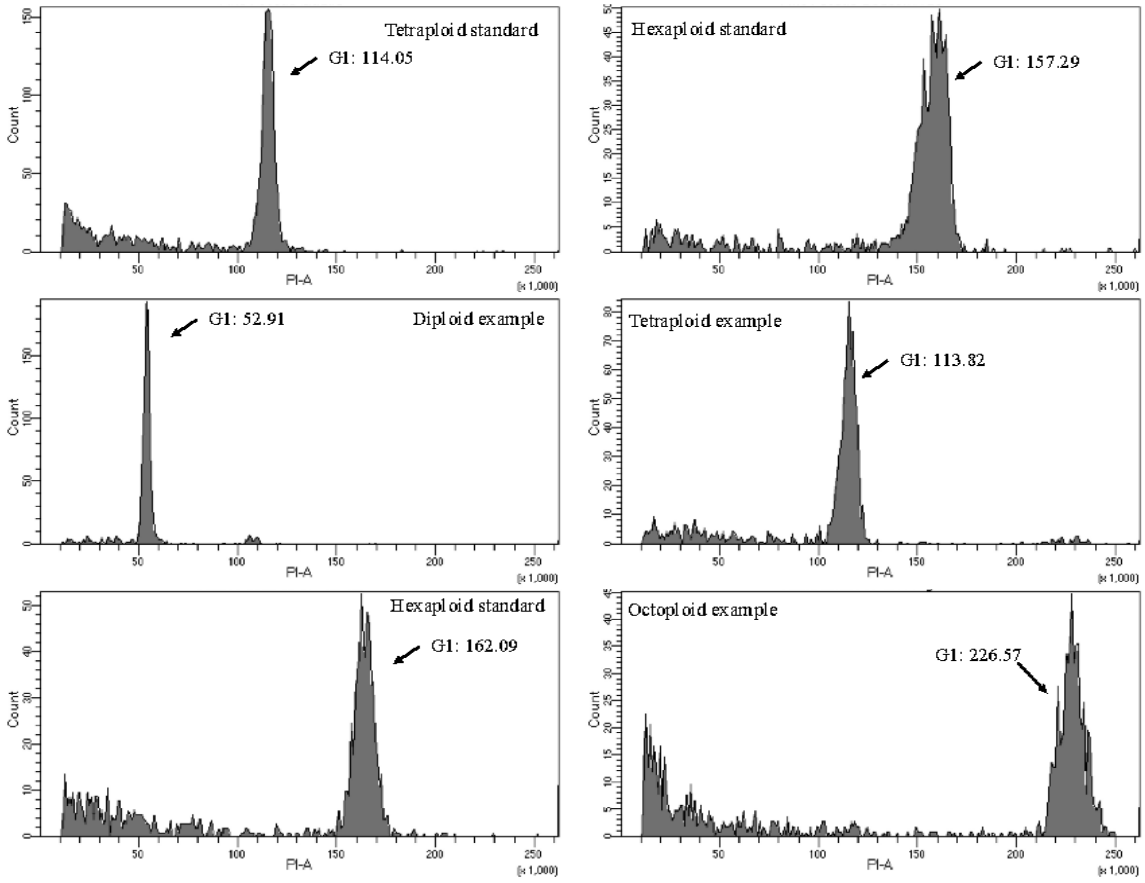
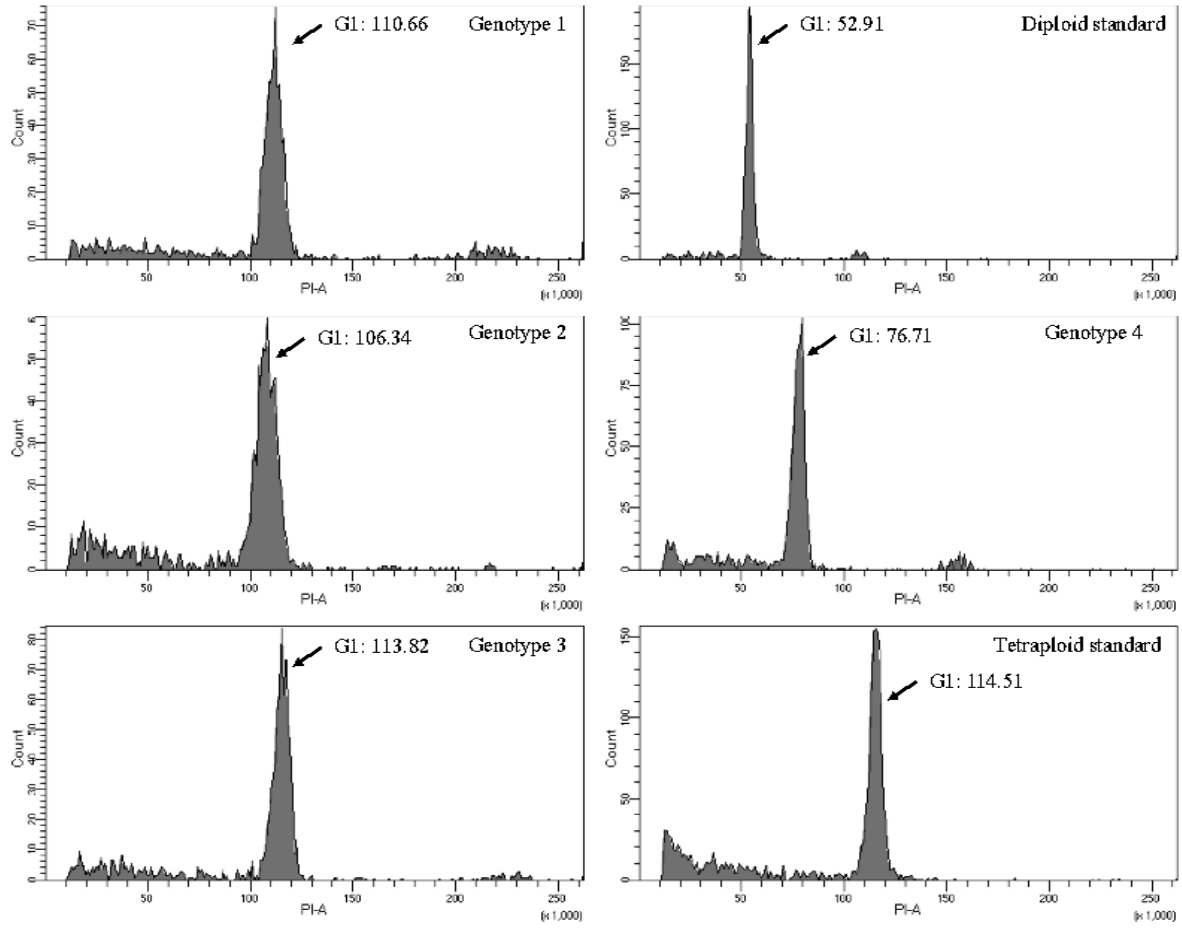


Figure 1. Flow cytometry data example of the PI accessions. Tetraploid sheep fescue cultivar 'Quatro' and hexaploid hard fescue cultivar 'Beacon' were included as standards. PI accessions included taxa that cover at least four ploidy levels.

149 The largest standard deviation within accession was 1.25 pg (PI 274619) while the lowest
150 was 0.01 pg (W6 23622). The average of standard deviation of the 126 accessions was 0.45 pg.
151 The majority of accessions had consistent DNA content (judged by standard deviation) within
152 the three genotypes examined. There were cases in which the sampled genotypes had more than
153 one ploidy level. For example, for PI 330706, there were two tetraploids and one triploid plant;
154 however, the fourth sample suggested the accession was tetraploid (**Figure 2**). Three out of the
155 five genotypes examined in PI 235072 accession had three different ploidies (data not shown)
156 and were therefore excluded from further analysis. The DNA content estimation of the 126
157 accessions is summarized in **Figure 3**.



158

Figure 2. Flow cytometry histogram of PI 330706. Three genotypes from this accession had similar DNA content compared to the tetraploid standard cultivar 'Quatro'. One genotype from this accession had a DNA content between diploid and tetraploid standard and was estimated to be triploid based on the DNA content estimation.

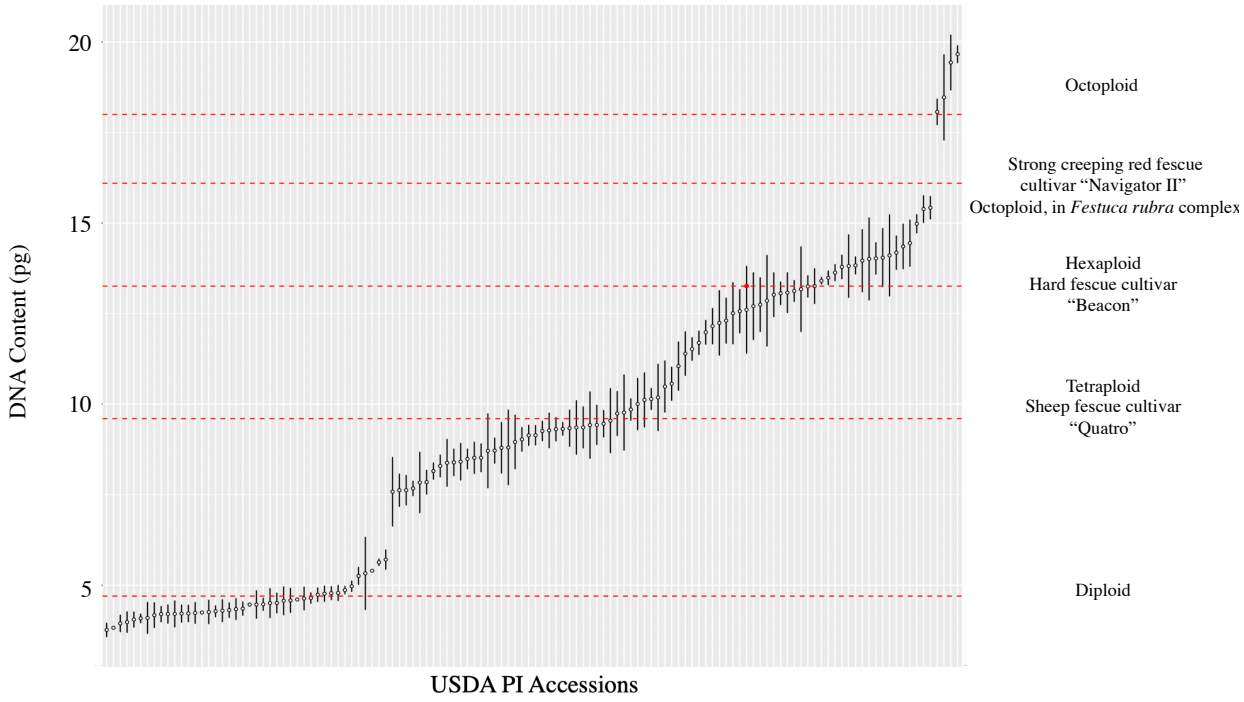


Figure 3. Accessions sorted by the estimated DNA content. Dashed red lines represent the ploidy level estimation for cultivars listed to the right of the figure.

Ploidy Estimation

To estimate ploidy, estimated DNA content was divided by the DNA content of the known diploid and round up to an integer. Ploidy estimates for the 126 accessions are summarized in **Table 1**. The majority of the accessions (82%) were di-, tetra-, hexa-, and octoploids while 18% of accessions were potential tri-, penta-, and septaploids. The seven accessions estimated as triploid had the estimated ploidy level between 3.23-3.47; the 14

pentaploid accessions had estimated ploidy between 4.70-5.47; and the 2 septaploids had estimated ploidy level of 6.55 and 6.56. Ploidy distribution by country of origin is shown in **Figure 4**.

Table 1. The ploidy level estimation of the 126 USDA PI accessions.

Ploidy level	Diploid	Tetraploid	Hexaploid	Octoploid	Other Ploidy
Accession Count	42	34	22	4	24
DNA content range (pg)	3.76 ~ 5.71	8.29 ~ 10.48	13.02~ 14.98	18.07 ~ 19.66	NA

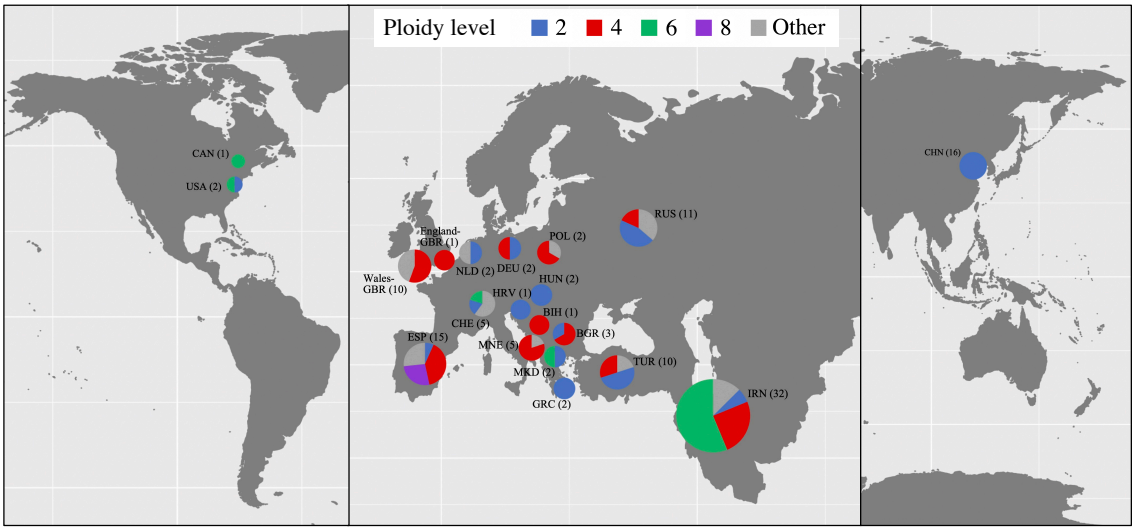
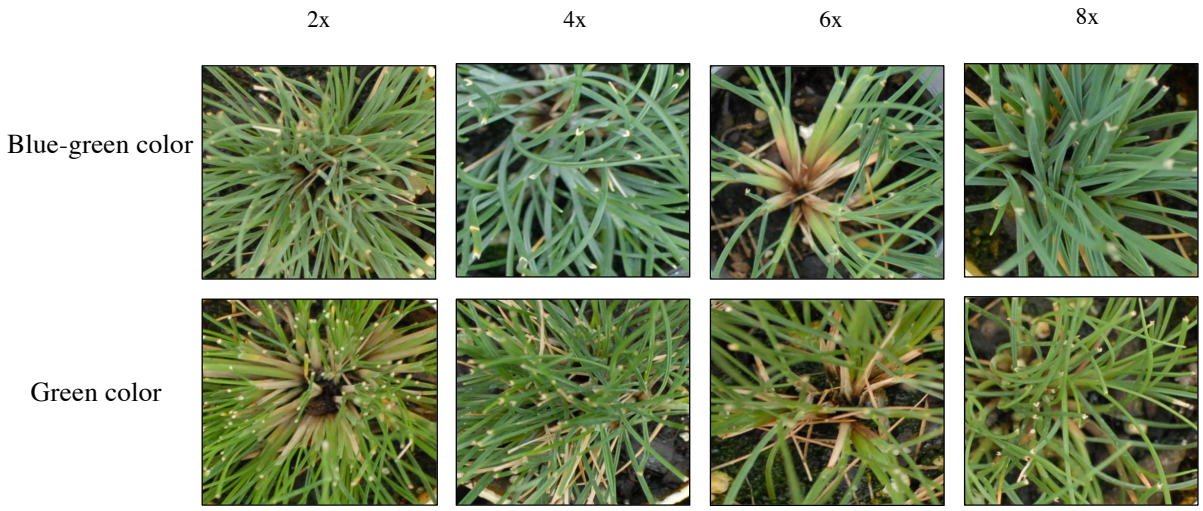


Figure 4. Ploidy estimation by DNA content for the 126 USDA PI accessions.

Besides DNA content variation, we observed leaf color variation at different ploidy levels. For all ploidy levels, there were the presence of blue-greenish color and green color

182 (**Figure 5**).



183

184 **Figure 5**. Leaf color differences observed in the *F. ovina* collection. We observed both green and

185 blue-greenish leaf color for plants at all four ploidy levels.

186 **Seed Size and Ploidy**

187 We found statistical differences in seed size among ploidy levels. In general, higher

188 ploidy level was associated with larger seed size. Diploid accessions had the average seed size

189 between 1.5 and 2.5 mm^2, tetraploid accessions seed size varies between 2 and 4 mm^2, and

190 hexaploid and octoploid had similar seed size between 4-6.5 mm^2 (**Figure 6**).

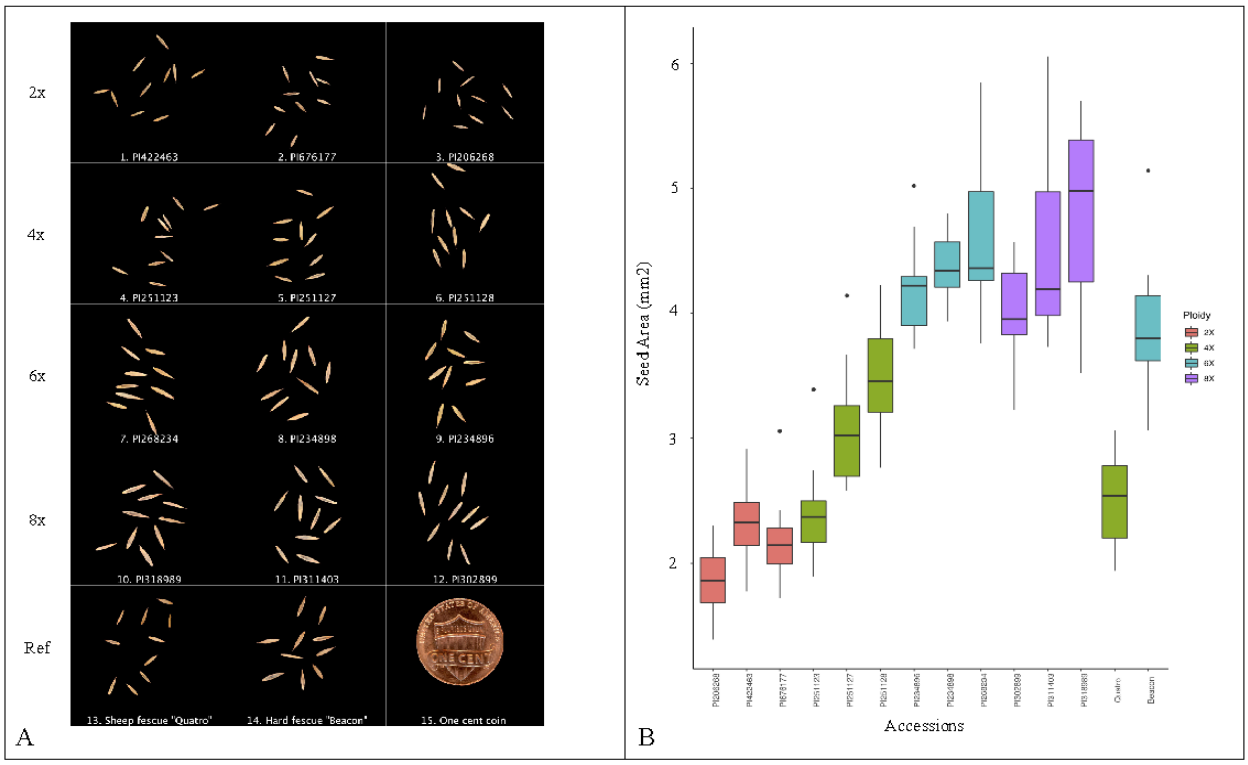


Figure 6. Comparison of seed area and ploidy level. The seed morphology of the examined 12 accessions and two fine fescues cultivars were shown in (A) with the ploidy of each accession labeled on the left. Box plot of seed size comparison is shown in (B); larger seed size was associated with higher ploidy level.

Analysis of the main effects model suggested that the ploidy level explains over 70% of the variation in seed area and length, and more than 55% of the variation in seed length. We rejected the hypothesis that ploidy level has no significant effect on the seed size of fine fescues (*p-value*: < 2.2e-16) (**Table S3, S4)**.

DISCUSSION

Accessions from the USDA NPGS can serve a valuable role as a source of genetic diversity for crop improvement providing an important gene pool for the improvement of both abiotic and biotic stress tolerance (Rubenstein, Smale, & Widrlechner, 2006). Numerous studies have been done to characterize PI germplasm with topics varying from soybean maturity groups (Nelson et al., 1987) to the identification of allelopathic accessions in rice (Dilday et al., 1994). These accessions have also been used to selected for reproductive characteristics and seed production in garlic (Jenderek & Hannan, 2004), screening for disease resistance in dry beans (Pastor-Corrales, 2003), and the evaluation of drought tolerance in watermelon at seedling stages (Zhang et al., 2011). In forage crops and turfgrasses, tall wheatgrass was evaluated for forage yield and quality (Vogel & Moore, 1998) and Kentucky bluegrass was studied for the reproductive mode (Wieners, Fei, & Johnson, 2006). It is clear that USDA PI accessions provide a diverse gene pool for cultivar abiotic and biotic stress tolerance improvement (Rubenstein et al., 2006). The USDA accessions are particularly important for turfgrass breeding and genetics because commercial turfgrass cultivars have undergone heavy selection and lost genetic diversity.

Different from major crops, most turfgrass species have numerous ploidy levels and some are morphologically indistinguishable; therefore, germplasm characterization is very important. Ploidy determination of 200 perennial ryegrass (*Lolium perenne*) suggested that six accessions were tetraploid and 194 were diploid (Wang, Bigelow, & Jiang, 2009). Screening of buffelgrass (*Cenchrus ciliaris* L. syn. *Pennisetum* ciliare (L.) Link) germplasm suggested multiple ploidy levels existed in the PI collection (Burson, Actkinson, Hussey, & Jessup, 2012). A similar result

223 was found in buffalograss, where multiple ploidy levels were found in the PI collections
224 (Johnson et al., 1998).

225 The *F. ovina* complex includes seven species and three additional subspecies that are
226 highly outcrossing and visually indistinguishable (Stace, 2010; Watson, 1958; Wilkinson &
227 Stace, 1991). The 126 *F. ovina* USDA PI accessions used in our study have ploidy levels ranging
228 from diploid to octoploid. The DNA content predicted by flow cytometry showed low average
229 standard deviation, suggesting the methods for DNA content measurement is consistent. We
230 measured DNA content variation between genotypes at same ploidy level, suggesting complex
231 genome composition variation, which could be explained by post-polyploid diploidization events
232 with differential gene loss (Mandáková & Lysak, 2018). This variation could also be the result of
233 the change of transposon elements (Kidwell, 2002), and hybridization in the field (Leitch &
234 Leitch, 2008; Soltis, Marchant, Van de Peer, & Soltis, 2015) or different chromosome size in
235 different populations (Ceccarelli, Falistocco, & Cionini, 1992).

236 Although the DNA content of diploid samples was clearly separated from higher ploidies,
237 there is no clear separation between accessions with higher ploidy levels. We also noticed ploidy
238 level variation within some accessions. For example, of the four genotypes within the accession
239 PI 330706 examined in this study, three were tetraploid while one was triploid. The triploid plant
240 is likely the result of hybridization between the tetraploid plant with the pollen from some
241 diploid relative. Another example is PI 235072, where we found three ploidy levels represented
242 in the five genotypes we examined. It is known that *F. ovina* species can easily hybridize with
243 relatives even at different ploidy levels (Jenkin & Jenkin, 1955). Under open pollination
244 conditions, it is not surprising that the seed purity is low. Our results suggest that resources

should be allocated such that the relevant USDA NPGS center managing open-pollinated species can properly isolate collections during seed increase.

While most accessions we surveyed can be assigned to discrete ploidy bins, 18% suggested DNA content that fell between ploidy levels. These accessions may have originated through hybridization between different diploid parents. It is also possible that these accessions are either aneuploid or dysploid, both of which have experienced chromosome gain/loss, or rearrangements. Further evaluation using a number of different approaches, ranging from morphological classification to genotyping, will be needed to fully classify these accessions.

In our study, all 16 accessions from China were found to be diploids and majority of plants from Iran were hexaploid. It is known that environmental and geographical factors played a role in *Festuca* ssp. genome size evolution (Ceccarelli et al., 1992; Šmarda, Bureš, Horová, Foggi, & Rossi, 2008). It would be interesting to see if the geographic location would be a factor to explain the distribution of ploidy levels. However, there is a lack of information on the specific area each wild accession was collected. Geographic information added to each accession in the NPGS collection would be useful; Rubenstein et al. (2006) found that NPGS users were more likely to utilize accessions when additional and accurate information was given about accessions. Beyond plant breeding, this new knowledge about the *F. ovina* collection might inspire other avenues of exploration such as investigating how geographic origin plays a role in taxon adaptation.

Seed size comparison suggests that accessions with higher ploidy levels tend to have a bigger seed size in *F. ovina* complex. Seed size often has an important impact on germination and plant development. Bretagnolle et al. (1995) found that larger seed size is correlated with

higher ploidy level in *Dactylis glomerata* L. and the larger seed size had a positive influence on robust seedling growth (Bretagnolle, Thompson, & Lumaret, 1995). Larger seeds contain more carbohydrates that provide the seedling vigor to help increase the competitive advantage in the natural environment (Te Beest et al., 2011). Besides observing DNA content variation, we also observed phenotypic variation on leaf color within the *F. ovina* collection. Green and bluish leaf color was observed in all ploidy levels, suggesting that leaf-color-based fine fescue identification is not reliable (Beard, 1972; Hubbard, 1968).

CONCLUSION

We evaluated 127 USDA PI accession and provided their DNA content and ploidy level estimation for 126 accessions. A total of 102 accessions were assigned to discrete ploidy levels, with the remaining had DNA content between discrete ploidy levels. Because of the cross-pollinating nature of *Festuca ovina* complex, better pollen control during the germplasm maintenance period could potentially reduce the chance of contaminations. Meanwhile, researchers should examine the PI collections to determine their ploidy level prior to adapting the accessions in their breeding program. This research builds the ground work for turfgrass researchers for using the *F. ovina* in their breeding program.

CONFLICT OF INTEREST STATEMENT

The authors declare there are no conflicts of interest.

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295 **SUPPLEMENTAL MATERIAL**

296 **Table S1.** USDA PI collections by the country of origins. Accessions used in this study covered

297 20 countries, with Iran having the most entries.

Location	PI Number					
Bosnia and Herzegovina	251128					
Bulgaria	634302	634303	636567			
Canada	236832					
China	499640	595130	595140	595145	595146	595158
	618975	595167	595170	595178	618972	634304
	655206	W6 23550	W6 23594	W6 23622		
Croatia	251421					
England	595052					
Germany	237708	422463				
Greece	206561	249739				
Hungary	257740	257741				
Iran	227362	227506	227507	229453	229454	229533
	229456	229497	229502	229503	230247	
	251384	251385	268234	330706	380845	380846
	380847	380848	380849	380850	380851	380852
	380853	380854	380855	380856	380857	384860
	384861	384863	547398			
Macedonia	250965	250967				
Montenegro	251123	251125	251126	251127	251131	
Netherlands	237179	315448				
Poland	274619	283320	287541			
Russia	115358	312453	314522	314523	314571	314687
	316249	371896	538933	538934	676177	
Spain	234478	234750	234751	234752	234758	287822
	287823	289652	302898	302899	302900	311403
	311405	318989	318990			
Switzerland	234895	234896	234897	234898	235072	
Turkey	109497	206268	340103	383650	383651	383652
	383653	383654	383655	568183		
United States	578733	537103				
Wales	577098	577099	595049	595050	595051	595059

	595060	595061	595062	595063
Unknown	189146			

298

299

300 **Table S2**. DNA content estimation and standard deviation of the USDA PI accessions. Data was
301 sorted by the DNA content from the smallest to the largest.

PI number	Average DNA Content (pg)	DNA Content SD (pg)	Estimated ploidy level
109497	4.57	0.39	2
115358	3.77	0.19	2
189146	4.61	0.01	2
206268	4.51	0.40	2
206561	4.31	0.20	2
227362	9.77	1.04	4
227506	8.52	0.39	4
227507	8.49	0.27	4
229453	9.54	0.89	4
229454	8.39	0.37	4
229456	14.18	0.46	6
229497	8.72	0.35	4
229502	8.80	1.03	4
229503	14.45	0.64	6
229533	13.02	0.61	6
230247	13.26	0.48	6
234478	4.58	0.33	2
234750	18.07	0.36	8
234751	12.51	0.85	5 (5.32)
234752	8.80	0.70	4
234758	9.74	0.61	4
234895	11.52	0.31	5 (4.90)
234896	14.02	0.44	6
234897	4.20	0.26	2
234898	15.42	0.31	7 (6.56)
236832	13.06	0.31	6
237179	4.22	0.24	2
237708	9.14	0.28	4
249739	4.78	0.18	2
250965	3.94	0.23	2
250967	13.41	0.09	6
251123	9.32	0.18	4
251125	10.18	0.92	4
251126	12.15	0.49	5 (5.17)
251127	10.00	0.71	4
251128	9.14	0.27	4

251131	10.12	0.75	4
251384	4.97	0.15	2
251385	13.96	0.86	6
251421	4.74	0.19	2
257740	4.20	0.21	2
257741	4.64	0.15	2
268234	14.01	1.13	6
274619	12.86	1.25	5 (5.47)
283320	10.14	0.29	4
287541	10.56	0.46	4
287822	9.42	0.92	4
287823	9.36	0.57	4
289652	7.67	0.20	3 (3.27)
302898	9.31	0.32	4
302899	19.66	0.24	8
302900	9.03	0.33	4
311403	18.47	1.18	8
311405	12.61	1.20	5 (5.36)
312453	10.49	0.71	4
314522	12.75	0.74	5 (5.43)
314523	12.71	0.92	5 (5.41)
314571	4.28	0.15	2
314687	4.26	0.32	2
315448	11.98	0.33	5 (5.10)
316249	8.96	0.74	4
318989	19.43	0.76	8
318990	11.05	0.67	5 (4.70)
330706	9.43	0.54	4
340103	9.26	0.27	4
371896	12.57	0.60	5 (5.35)
380845	13.63	0.23	6
380846	13.25	0.30	6
380847	12.24	0.89	5 (5.21)
380848	13.13	0.29	6
380849	15.39	0.37	7 (6.55)
380850	12.31	0.62	5 (5.24)
380851	13.79	0.33	6
380852	14.05	0.80	6
380853	14.35	0.62	6

380854	13.81	0.86	6
380855	14.11	1.12	6
380856	4.77	0.21	2
380857	13.83	0.24	6
383650	9.46	0.36	4
383651	4.87	0.10	2
383652	4.47	0.38	2
383653	8.15	0.23	3 (3.47)
383654	8.38	0.65	4
383655	7.85	0.33	3 (3.34)
384860	11.40	0.60	5 (4.85)
384861	13.08	0.55	6
384863	14.98	0.25	6
422463	4.21	0.36	2
499640	4.35	0.18	2
537103	13.17	1.17	6
538933	11.69	0.32	5 (4.98)
538934	4.17	0.34	2
547398	13.49	0.19	6
568183	4.22	0.23	2
577098	7.62	0.45	3 (3.24)
577099	7.84	0.84	3 (3.33)
578733	4.79	0.21	2
595049	7.62	0.41	3 (3.24)
595050	9.27	0.48	4
595051	9.85	0.30	4
595052	9.34	0.50	4
595059	7.58	0.95	3 (3.23)
595060	8.41	0.51	4
595061	9.36	0.74	4
595062	8.71	1.02	4
595063	8.52	0.43	4
595130	4.10	0.43	2
595140	4.30	0.30	2
595145	4.05	0.21	2
595146	3.82	0.04	2
595158	4.23	0.29	2
595167	4.25	0.04	2
595170	4.47	0.03	2

595178	4.34	0.29	2
618972	5.26	0.24	2
618975	4.08	0.12	2
634302	8.29	0.30	4
634303	4.47	0.17	2
634304	4.51	0.27	2
636567	3.98	0.28	2
655206	5.33	1.00	2
676177	4.63	0.32	2
W6 23550	5.71	0.27	2
W6 23594	5.63	0.09	2
W6 23622	5.40	0.01	2

302

303

Table S3. The linear regression to associate seed size with ploidy levels. *t* statistics suggested there was a significant difference in seed size between different ploidy levels.

Estimate	Std.	Error	*t* value	Pr (>|*t*|)
(Intercept)	2.1199	0.1079	19.644	< 2.00E-16 ***
Ploidy 4X	0.7584	0.1428	5.312	4.31E-07 ***
Ploidy 6X	2.1432	0.1428	15.013	< 2.00E-16 ***
Ploidy 8X	2.3051	0.1526	15.104	< 2.00E-16 ***

Signif. codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

Table S4. The ANOVA analysis of PI accessions using ploidy levels as the variable. Significant differences were found between seed size and the corresponding ploidy level.

Df	Sum	Sq	Mean	Sq	F- value	Pr(>F)
Ploidy	3	121.102	40.367	1.16E+02	< 2.20E-16	***
Residuals	136	47.514	0.349			

Signif. codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

AUTHOR CONTRIBUTIONS

YQ and SH performed the flow cytometry experiments, analyzed the data. JO performed the image analysis. YQ wrote the manuscript. EW secured funding for this project, supervised this research, provided suggestions, and comments. All authors contributed to the revision of the manuscript and approved the final version.

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