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

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

3 **Authors**: Yinjie Qiu*, Sierra Hamernick, Joan Barreto Ortiz, and Eric Watkins

4 *Corresponding Author: Yinjie Qiu, 440 Alderman Hall, Folwell Ave, St. Paul, MN 55108,

5 USA, qiuxx221@umn.edu

6 Y. Qiu, S.H, J.O, and E. Watkins, Department of Horticultural Science, University of Minnesota,

7 St. Paul, MN, 55108, USA

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

9 Germplasm System;

10

**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

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

68 For NPGS accessions from the *F. ovina* complex, it is necessary to determine ploidy level
69 before conducting germplasm selection and performing hybridization; this has traditionally been
70 done by counting chromosomes (Maluszynska, 2003; Vargas, McAllister, Morton, Jury, &
71 Wilkinson, 1999), a reliable but time-consuming method which is made all the more challenging
72 when dealing with the numerous and small (even under magnification) chromosomes of the fine
73 fescue species. Flow cytometry is a powerful tool for DNA content measurement and allows
74 researchers to estimate the ploidy level by comparing to known standards. This method is less
75 time consuming, cheaper, and proven to work in grasses where it has been used to calculate the
76 DNA content and estimate ploidy levels in Texas bluegrass (*Poa arachnifera* Torr.), buffalograss
77 [*Bouteloua dactyloides* (Nutt.) Engelm.], perennial ryegrass (*Lolium perenne* L.) and fine
78 fescues (Ka Arumuganathan & Earle, 1991; Goldman, 2015; Huff & Palazzo, 1998; Johnson,
79 Kenworthy, Auld, & Riordan, 2001; Johnson, Riordan, & Arumuganathan, 1998; Qiu et al.,
80 2019).

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

84

## MATERIAL AND METHODS

### Plant Material

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

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

### Flow Cytometry Procedure

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

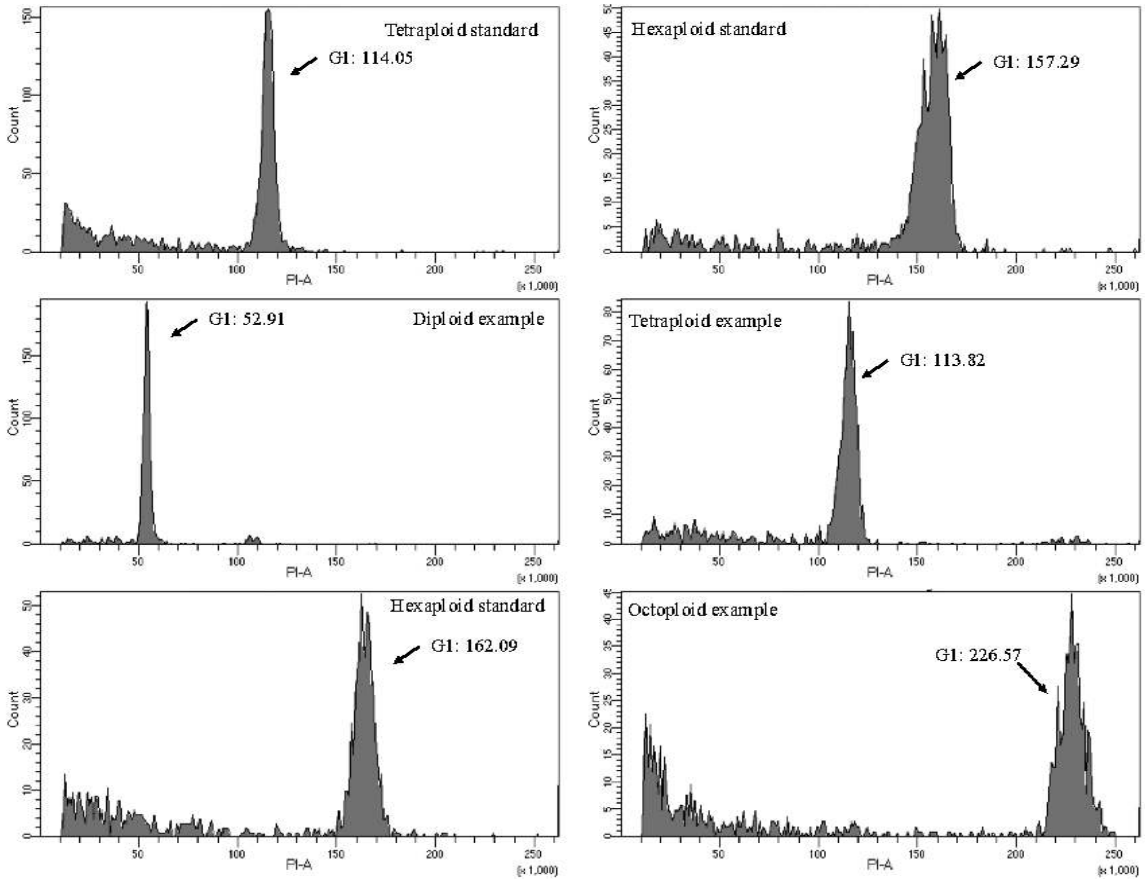
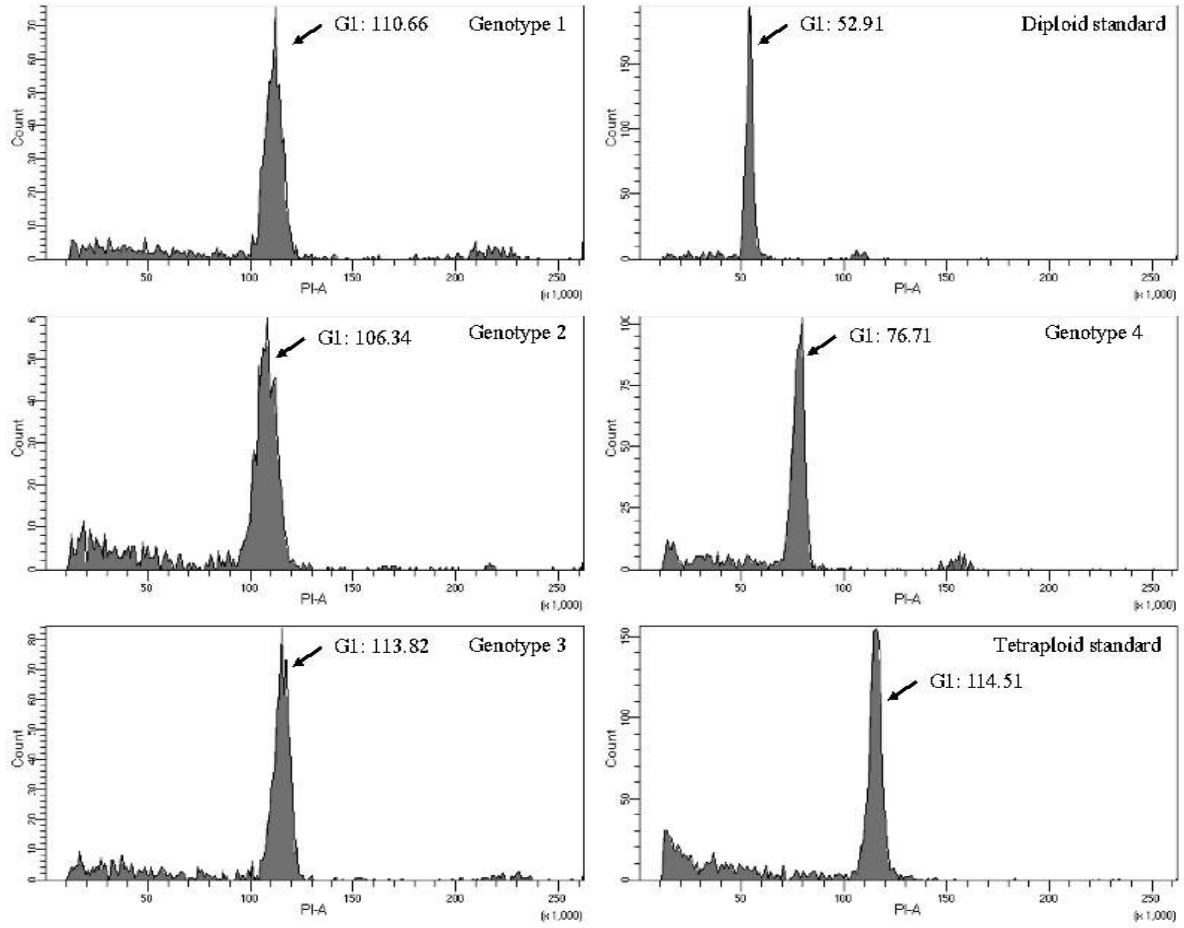


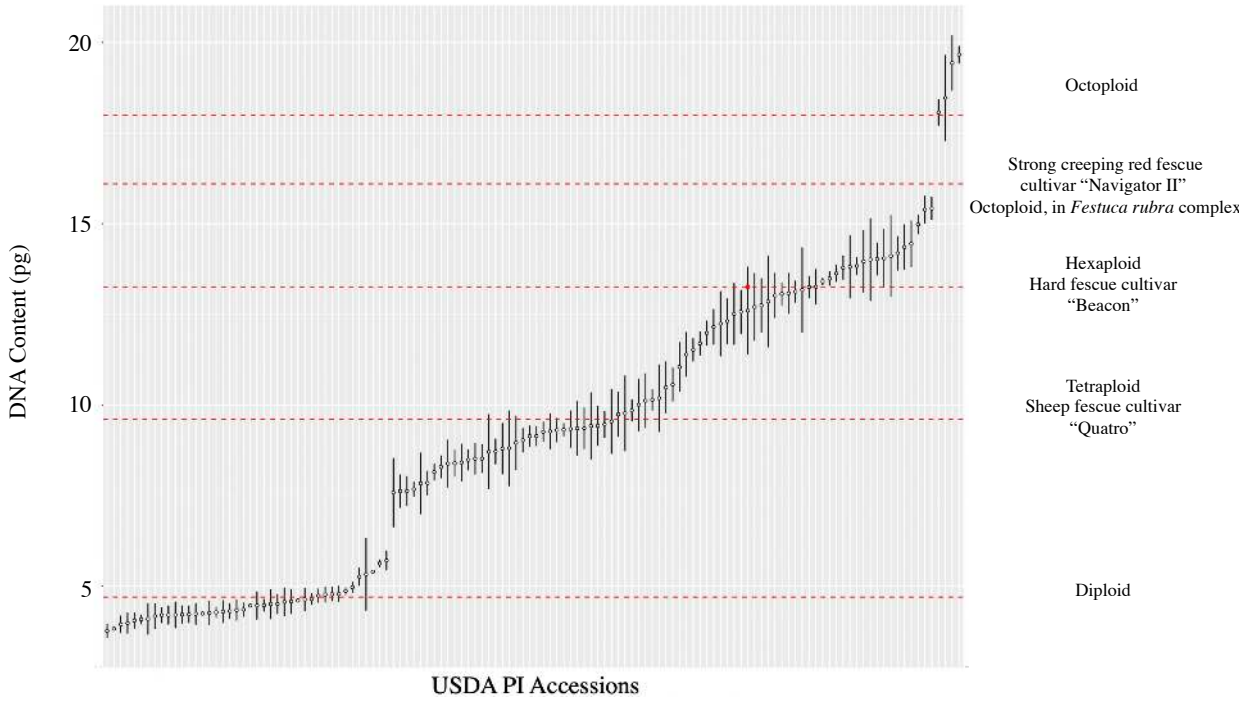

**Figure 1.** Flow cytometry data example of the PI accessions. Tetraploid sheep fescue cultivar 'Quatro' and hexaploid hard fescue cultivar 'Beacon' were included as standards. PI accessions included taxa that cover at least four ploidy levels.

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

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




163

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

166 **Ploidy Estimation**

167 To estimate ploidy, estimated DNA content was divided by the DNA content of the
168 known diploid and round up to an integer. Ploidy estimates for the 126 accessions are
169 summarized in **Table 1**. The majority of the accessions (82%) were di-, tetra-, hexa-, and
170 octoploids while 18% of accessions were potential tri-, penta-, and septaploids. The seven
171 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 |

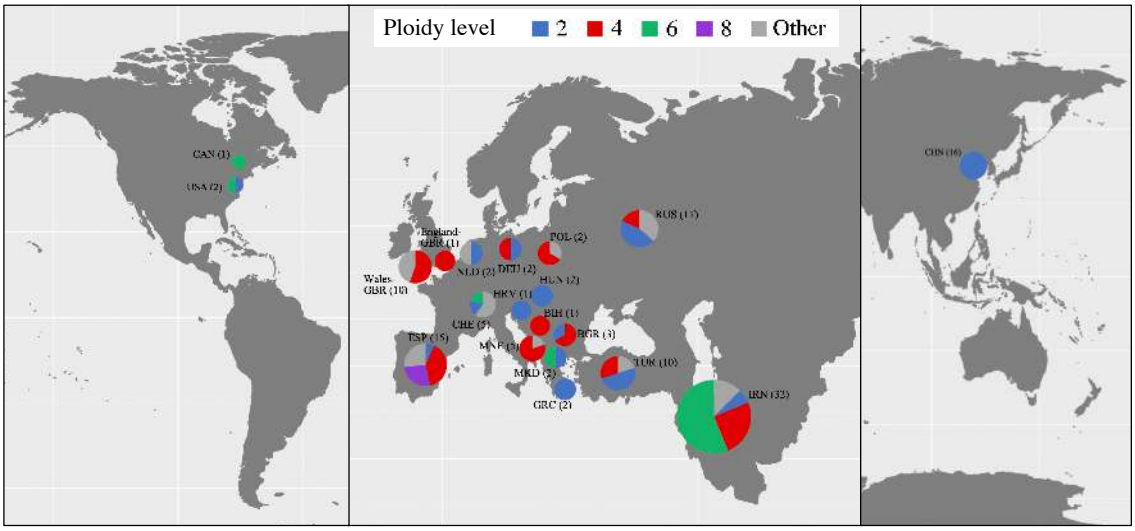
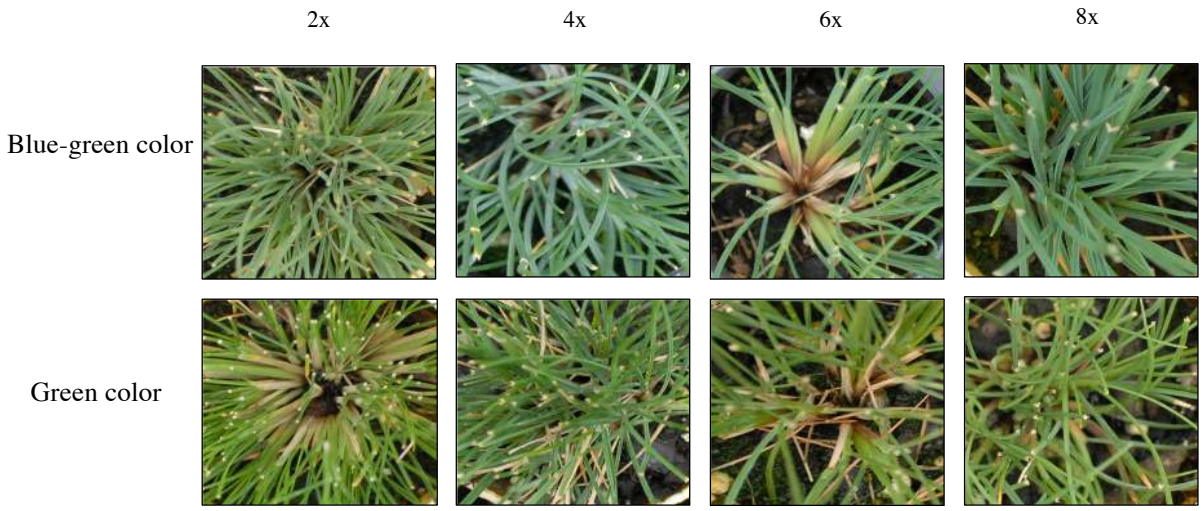



**Figure 4**. Ploidy estimation by DNA content for the 126 USDA PI accessions.

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**).

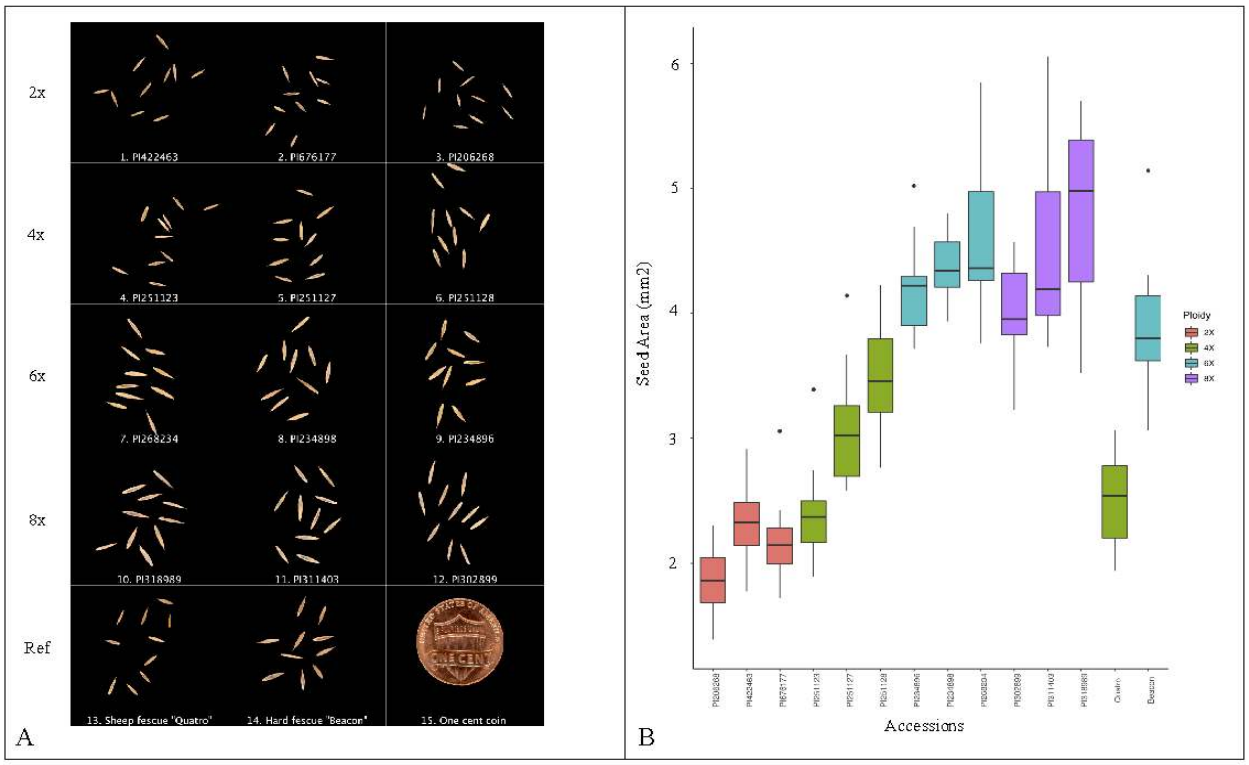



**Figure 6**. Comparison of seed area and ploidy level. The seed morphology of the examined 12 accessions and two fine fescues cultivars were shown in (A) with the ploidy of each accession labeled on the left. Box plot of seed size comparison is shown in (B); larger seed size was associated with higher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

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

274 **CONCLUSION**

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

283

## CONFLICT OF INTEREST STATEMENT

The authors declare there are no conflicts of interest.

## ACKNOWLEDGMENT

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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 |

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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.

316 **REFERENCES**

317 Arumuganathan, K., & Earle, E. (1991). Nuclear DNA content of some important plant species.
318 *Plant molecular biology reporter, 9*(3), 208-218.
319 Arumuganathan, K., Tallury, S., Fraser, M., Bruneau, A., & Qu, R. (1999). Nuclear DNA
320 content of thirteen turfgrass species by flow cytometry. *Crop Science, 39*(5), 1518-1521.
321 Baird, J. H., Kopecký, D., Lukaszewski, A. J., Green, R. L., Bartoš, J., & Doležel, J. (2012).
322 Genetic diversity of turf-type tall fescue using diversity arrays technology. *Crop Science,*
323 *52*(1), 408-412.
324 Barkworth, M., Capels, K., Long, S., Anderton, L., & Piep, M. (2007). Magnoliophyta
325 Commelinidae (in part: Poaceae, part 1). Flora of North America North of Mexico. Vol.
326 24. In: Oxford University Press: New York.
327 Beard, J. B. (1972). Turfgrass: Science and culture.
328 Bonos, S. A., Clarke, B. B., & Meyer, W. A. (2006). Breeding for disease resistance in the major
329 cool-season turfgrasses. *Annu. Rev. Phytopathol., 44*, 213-234.
330 Bonos, S. A., & Huff, D. R. (2013). Cool-season grasses: Biology and breeding. *Turfgrass:*
331 *Biology, use, and management* (turfgrassbiolog), 591-660.
332 Bretagnolle, F., Thompson, J., & Lumaret, R. (1995). The influence of seed size variation on
333 seed germination and seedling vigour in diploid and tetraploid *Dactylis glomerata* L.
334 *Annals of botany, 76*(6), 607-615.
335 Budak, H., Shearman, R., Gaussoin, R. E., & Dweikat, I. (2004). Application of sequence-related
336 amplified polymorphism markers for characterization of turfgrass species. *HortScience,*
337 *39*(5), 955-958.
338 Burson, B., Actkinson, J., Hussey, M., & Jessup, R. (2012). Ploidy determination of buffel grass
339 accessions in the USDA National Plant Germplasm System collection by flow cytometry.
340 *South African Journal of Botany, 79*, 91-95.
341 Casler, M. D. (2003). *Turfgrass biology, genetics, and breeding*: John Wiley & Sons.
342 Ceccarelli, M., Falistocco, E., & Cionini, P. (1992). Variation of genome size and organization
343 within hexaploid Festuca arundinacea. *Theoretical and Applied Genetics, 83*(3), 273-278.
344 Chang, H.-X., & Hartman, G. L. (2017). Characterization of insect resistance loci in the USDA
345 soybean germplasm collection using genome-wide association studies. *Frontiers in plant*
346 *science, 8*, 670.
347 Chen, Z., Wang, M. L., Waltz, C., & Raymer, P. (2009). Genetic diversity of warm-season
348 turfgrass: seashore paspalum, bermudagrass, and zoysiagrass revealed by AFLPs.
349 *Floriculture Ornamental Biotechnol, 3*(1), 20-24.
350 Christensen, S., Pratt, D. B., Pratt, C., Nelson, P., Stevens, M., Jellen, E. N., . . . Maughan, P. J.
351 (2007). Assessment of genetic diversity in the USDA and CIP-FAO international nursery
352 collections of quinoa (*Chenopodium quinoa* Willd.) using microsatellite markers. *Plant*
353 *Genetic Resources, 5*(2), 82-95.
354 Christiansen, M., Andersen, S. B., & Ortiz, R. (2002). Diversity changes in an intensively bred
355 wheat germplasm during the 20 th century. *Molecular Breeding, 9*(1), 1-11.
356 Clarke, B. B., White Jr, J. F., Hurley, R. H., Torres, M. S., Sun, S., & Huff, D. R. (2006).
357 Endophyte-mediated suppression of dollar spot disease in fine fescues. *Plant disease,*
358 *90*(8), 994-998.
359 Dilday, R., Lin, J., & Yan, W. (1994). Identification of allelopathy in the USDA-ARS rice
360 germplasm collection. *Australian Journal of Experimental Agriculture, 34*(7), 907-910.

361 Doležel, J., & Bartoš, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome
362 size. *Annals of botany, 95*(1), 99-110.
363 Goldman, J. J. (2015). DNA contents in Texas bluegrass (*Poa arachnifera*) selected in Texas and
364 Oklahoma determined by flow cytometry. *Genetic Resources and Crop Evolution, 62*(5),
365 643-647.
366 Goodman, M. M. (1999). Broadening the genetic diversity in maize breeding by use of exotic
367 germplasm. *The genetics and exploitation of heterosis in crops*(thegeneticsande), 139-
368 148.
369 Hubbard, C. E. (1968). Grasses. A guide to their structure, identification, uses, and distribution in
370 the British Isles. *Grasses. A guide to their structure, identification, uses, and distribution*
371 *in the British Isles.*
372 Huff, D. R., & Palazzo, A. J. (1998). Fine fescue species determination by laser flow cytometry.
373 *Crop Science, 38*(2), 445-450.
374 Jenderek, M. M., & Hannan, R. M. (2004). Variation in reproductive characteristics and seed
375 production in the USDA garlic germplasm collection. *HortScience, 39*(3), 485-488.
376 Jenkin, T., & Jenkin, T. (1955). Interspecific and intergeneric hybrids in herbage grasses-XIV.
377 The breeding affinities of *Festuca Ovina*. *Journal of Genetics, 53*(01).
378 Johnson, P. G., Kenworthy, K. E., Auld, D. L., & Riordan, T. P. (2001). Distribution of
379 buffalograss polyploid variation in the southern Great Plains. *Crop science, 41*(3), 909-
380 913.
381 Johnson, P. G., Riordan, T., & Arumuganathan, K. (1998). Ploidy level determinations in
382 buffalograss clones and populations. *Crop science, 38*(2), 478-482.
383 Kahle, D., & Wickham, H. (2013). ggmap: Spatial Visualization with ggplot2. *The R journal,*
384 *5*(1), 144-161.
385 Kidwell, M. G. (2002). Transposable elements and the evolution of genome size in eukaryotes.
386 *Genetica, 115*(1), 49-63.
387 Leitch, A., & Leitch, I. (2008). Genomic plasticity and the diversity of polyploid plants. *Science,*
388 *320*(5875), 481-483.
389 Leng, Y., Wang, R., Ali, S., Zhao, M., & Zhong, S. (2016). Sources and genetics of spot blotch
390 resistance to a new pathotype of Cochliobolus sativus in the USDA National small grains
391 collection. *Plant disease, 100*(10), 1988-1993.
392 Maluszynska, J. (2003). Cytogenetic tests for ploidy level analyses—chromosome counting. In
393 *Doubled haploid production in crop plants* (pp. 391-395): Springer.
394 Mandáková, T., & Lysak, M. A. (2018). Post-polyploid diploidization and diversification
395 through dysploid changes. *Current opinion in plant biology, 42*, 55-65.
396 Melchinger, A. (1999). Genetic diversity and heterosis. In (Vol. 10): chapter.
397 Mikel, M. A., Diers, B. W., Nelson, R. L., & Smith, H. H. (2010). Genetic diversity and
398 agronomic improvement of North American soybean germplasm. *Crop science, 50*(4),
399 1219-1229.
400 Nelson, R. L., Amdor, P. J., Orf, J. H., & Cavins, J. F. (1988). Evaluation of the USDA soybean
401 germplasm collection: maturity groups 000 to IV (PL 427.136 to Pl 445.845). *Technical*
402 *bulletin-United States Department of Agriculture*, (1726).
403 Pastor-Corrales, M. (2003). Sources, genes for resistance and pedigrees of 52 rust and mosaic
404 resistant dry bean germplasm lines released by the USDA Beltsville bean project in
405 collaboration. *Science, 29*(3), 834-835.

406 Piper, C. V. (1906). *North American species of Festuca* (Vol. 10): US Government Printing
407 Office.
408 Prasanna, B. (2012). Diversity in global maize germplasm: characterization and utilization.
409 *Journal of biosciences, 37*(5), 843-855.
410 Qiu, Y., Hirsch, C. D., Yang, Y., & Watkins, E. (2019). Towards Improved Molecular
411 Identification Tools in Fine Fescue (*Festuca* L., Poaceae) Turfgrasses: Nuclear Genome
412 Size, Ploidy, and Chloroplast Genome Sequencing. *Frontiers in Genetics, 10*(1223).
413 doi:10.3389/fgene.2019.01223
414 Reif, J. C., Zhang, P., Dreisigacker, S., Warburton, M. L., van Ginkel, M., Hoisington, D., . . .
415 Melchinger, A. E. (2005). Wheat genetic diversity trends during domestication and
416 breeding. *Theoretical and Applied Genetics, 110*(5), 859-864.
417 Rubenstein, K. D., Smale, M., & Widrlechner, M. P. (2006). Demand for genetic resources and
418 the US National Plant Germplasm System. *Crop Science, 46*(3), 1021-1031.
419 Ruemmele, B., Brilman, L., & Huff, D. (1995). Fine fescue germplasm diversity and
420 vulnerability. *Crop Science, 35*(2), 313-316.
421 Schmit, R., Duell, R., & Funk, C. (1974). *Isolation barriers and self-compatibility in selected*
422 *fine fescues 1.* Paper presented at the Proceedings of the Second International Turfgrass
423 Research Conference.
424 Seal, A. (1983). DNA variation in Festuca. *Heredity, 50*(3), 225.
425 Šmarda, P., Bureš, P., Horová, L., Foggi, B., & Rossi, G. (2008). Genome size and GC content
426 evolution of Festuca: ancestral expansion and subsequent reduction. *Annals of botany,*
427 *101*(3), 421-433.
428 Soltis, P. S., Marchant, D. B., Van de Peer, Y., & Soltis, D. E. (2015). Polyploidy and genome
429 evolution in plants. *Current opinion in genetics & development, 35*, 119-125.
430 Stace, C. (2010). *New flora of the British Isles*: Cambridge University Press.
431 Te Beest, M., Le Roux, J. J., Richardson, D. M., Brysting, A. K., Suda, J., Kubešová, M., &
432 Pyšek, P. (2011). The more the better? The role of polyploidy in facilitating plant
433 invasions. *Annals of botany, 109*(1), 19-45.
434 Van Esbroeck, G., & Bowman, D. T. (1998). Cotton germplasm diversity and its importance to
435 cultivar development. *Journal of Cotton Science*.
436 Vargas, P., McAllister, H. A., Morton, C., Jury, S. L., & Wilkinson, M. J. (1999). Polyploid
437 speciation inHedera (Araliaceae): Phylogenetic and biogeographic insights based on
438 chromosome counts and ITS sequences. *Plant Systematics and Evolution, 219*(3-4), 165-
439 179.
440 Vasey, G. (1883). *Grasses of the United States*: US Government Printing Office.
441 Vogel, K. P., & Moore, K. J. (1998). Forage yield and quality of tall wheatgrass accessions in the
442 USDA germplasm collection. *Crop science, 38*(2), 509-512.
443 Wang, Y., Bigelow, C. A., & Jiang, Y. (2009). Ploidy level and DNA content of perennial
444 ryegrass germplasm as determined by flow cytometry. *HortScience, 44*(7), 2049-2052.
445 Watson, P. J. (1958). The distribution in Britain of diploid and tetraploid races within the Festuca
446 ovina group. *The New Phytologist, 57*(1), 11-18.
447 Wieners, R. R., Fei, S.-z., & Johnson, R. C. (2006). Characterization of a USDA Kentucky
448 bluegrass (*Poa pratensis* L.) core collection for reproductive mode and DNA content by
449 flow cytometry. *Genetic Resources and Crop Evolution, 53*(8), 1531-1541.

Wilkinson, M. J., & Stace, C. A. (1991). A new taxonomic treatment of the *Festuca ovina* L. aggregate (Poaceae) in the British Isles. *Botanical Journal of the Linnean Society, 106*(4), 347-397.

Zhang, H., Gong, G., Guo, S., Ren, Y., Xu, Y., & Ling, K.-S. (2011). Screening the USDA watermelon germplasm collection for drought tolerance at the seedling stage. *HortScience, 46*(9), 1245-1248.