A DIHAPLOID APPROACH FOR THE SELECTION OF FORAGE QUALITY IN TALL FESCUE (*FESTUCA ARUNDINACEA* SCHREB.)

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A B S T R A C T

Within the *Festuca-Lolium* genome complex, there is a need for modern breeding approaches that can facilitate the rapid development of improved germplasm or cultivars. Traditional recurrent or mass-selection methods for population or synthetic development are labor intensive and time consuming. The recent development of annual ryegrass *Lolium multiflorum* Lam.) lines, when hybridized by tall fescue [(*Festuca arundinacea* Schreb. (syn. = *Lolium arundinaceum* (Schreb.) Darbysh.)], will produce F1 hybrids, that through mitotic genome loss, lose either their ryegrass or tall fescue genome and result in the recovery of ryegrass and tall fescue dihaploid (DH) lines. For breeding and selection purposes regarding forage quality, the ability to select for superior genotypes at the F1 generation, rather than at the subsequent DH generation would improve the efficiency of the technique. The study examined forage quality components of crude protein (CP), acid digestible fiber (ADF), neutral digestible fiber (NDF) and in vitro dry matter digestibility (IVDMD) expression and inheritance in the F1 and their respective recovered DH lines. Results of the study indicate that forage quality genotypes, conferred by the tall fescue gamete, are heritable and selection performed at the F1 generation will be effective in identifying DH genotypes that are not significantly different in forage quality of their respective F1 parents. Though performed only on CP, ADF, NDF and IVDMD forage quality components, the study suggests similar application toward the selection of additional quantitative forage quality or agronomic attributes for tall fescue improvement. The described selection strategy is applicable to tall fescue (*L. arundinaceum*) and may have application across the *Lolium/Festuca* genus.

Keywords: dihaploid, tall fescue, forage, quality.

INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb. (syn. = *Lolium arundinaceum* (Schreb.) Darbysh.; *Schedonorus arundinaceus (Schreb. Dumort.*)), 2n=6x=42, represents the predominant, introduced perennial cool-season grass forage in the USA. Its wide adaptation, spring, summer, and fall forage production, deep root system, tolerance to heat and persistence make this a desirable species for hay, pasture and turf. It responds well to fertilizer but can maintain itself under limited fertility and is adapted to moderately acid and wet soils (Jennings *et al.*, 2008). Tall fescue is a self-incompatible, outbreeding allohexaploid that represents a species complex consisting of Continental, Mediterranean and rhizomatous (Hand *et al.*, 2010). Its diversity of genotypes varies in morphology, agronomic attributes, persistence, drought and other

agronomic attributes which have and are the focus of various breeding or selection schemes (Kasperbauer, 1990; Humphreys *et al.*, 1997; Volaire and Norton, 2006). However, a gamete selection approach has not been applicable due to the lack of an efficient dihaploid generation system. Recently, two *Lolium multiflorum* Lam. lines were released (IL1, IL2) (Kindiger and Singh, 2011; Kindiger, 2012a) that allow low levels of dihaploid generation in hybrids when using the IL lines as the maternal parent in crosses that utilize tall fescue as the paternal parent (Kindiger, 2012b, 2016). Briefly, with the utilized approach, the generated F1 will occasionally loose either the IL (ryegrass) genome; or, the tall fescue genome through a mitotic genome loss behavior. This behavior gives rise to viable eggs cells having only a

ryegrass or tall fescue genome. Both are receptive to fertilization. When such ovaries spontaneously double in chromosome number in the F1 inflorescence, a homozyougous, DH ryegrass (IL); or, a homozygous, tall fescue DH line are generated (Kindiger, 2012b, 2016).

Gamete selection, as originally defined by Stadler (1944), is based on the principle that selection exerted at the gametophytic level can increase desirable allelic frequencies detectable at the sporophytic level. Sporophytic expressed traits are transmitted as genetic information through the gametes (sperm or egg nuclei) and contain half the information that is contained in the sporophytic tissue. If superior gametes can be recognized through appropriate selection methods, then such a system would theoretically be more efficient than one based on a zygotic selection strategy (Richey, 1947). Through the utilization of a paternal monoploid or dihaploid generation process, gamete selection has been a proven and efficient method of selection across numerous species (Stadler, 1944; Fehr, 1984; Snape *et al.*, 1986; Schon *et al.*, 1990; Lu *et al.*, 1996; Rotarenco and Chalyk, 2000). In this methodology, the genetic contribution of the tall fescue gamete is the genotype of primary interest for tall fescue DH generation.

Dihaploid (DH) selection methods are well known for their ability to result in more rapid and efficient gains than other forms of selection (Singh, 1994; Hussain *et al.*, 2012). In addition, the effectiveness of a DH selection approach is elevated when the number of genes governing a particular trait as quantitative in their inheritance (Kotch *et al.*, 1992). The success of gamete selection is a more rapid breeding/selection approach and that no prior knowledge regarding the number of genes or inheritance of a trait is required (Singh, 1994). When applied to a polyploid species such as tall fescue, the gain in breeding efficiency could be exponential. Gamete selection, with early generation selection, has successfully been applied to the simultaneous selection of multiple traits, including quantitative trait loci governing characteristics such as seed yield, maturity, and tolerance to disease (Singh, 1994; Ravikumar and Patil, 2004). Though DH breeding methods, utilizing microspore tissue culture approaches, have been occasionally employed as a means to develop new Festulolium germplasm within the *Festuca-Lolium* complex of polyploid grasses (Humphreys *et al.*, 2003; Guo and Yamada, 2004; Guo *et al.*, 2005), the process is time consuming, tedious, genotypic specific and does not employ a gamete

selection strategy.

Early research that utilized homozygous tall fescue derivatives, as generated through a standard selfing program, previously examined the inheritance of palatability (Henson and Buckner, 1957; Buckner and Fergus, 1960). Though the inbreeding process was time consuming, these studies indicated that the selection and development of homozygous lines can represent a useful methodology for the improvement of tall fescue. The success of this research also indicates that the application of a gamete selection approach aligned with the production of DH lines (Bouchez and Gallais, 2000) could result in a more efficient breeding approach for the production of tall fescue cultivars.

In the gamete selection approach presented here, a slight modification of a method presented by Stadler (1944) is utilized. In practice, gamete selection ordinarily involves two steps: 1) selection on the basis of outcross performance testing of individual plants of a variety or populations; and 2) a similar controlled selection for outstanding individuals exhibiting desirable agronomic attributes. Following the identification of superior genotypes, such individuals would undergo continued selfing, followed by phenotypic selection to generate homozygous stocks fixed for the desired agronomic characteristics. In instances where haploids can be generated through microspore culture, followed by genome doubling or some other method to induce homozygosity, homozygous or DH lines are the result. As with the Stadler gamete selection approach, the described approach reflects the situation where traits transmitted through the gamete are under the control of genes that are expressed in the sporophyte. But, unlike Stadler (op. cit.), a single gamete from the tall fescue parent fertilizes the egg of the inducer line (IL) which produces the F1 hybrid. Selection applied upon the F1 sporophyte and the tall fescue genome that it possesses constitutes the described form of gamete selection. Following the mitotic loss of one genome or the other, parthenogenesis occurs producing a viable embryo, but a seed lacking an endosperm (Kindiger, 2016). These events generally result in tall fescue DH recoveries possessing a normal genome constitution ($2n=6x=42$) and occasional ryegrass DH recoveries possessing a normal, diploid genome constitution ($2n=2x=14$). The described research examines the inheritance and expression of CP, ADF, NDF and IVDMD from the F1 stage to the DH recovery. The approach constitutes a gamete based DH technology that

greatly enhances the production of homozygous DH lines and an understanding of the inheritance and expression of some forage quality attributes may identify a more efficient and effective approach for developing superior tall fescue DH lines and cultivars than traditional recurrent or mass-selection techniques.

MATERIALS AND METHODS

The USDA-ARS has recently released two annual ryegrass genetic stocks, identified as IL1 and IL2 (Kindiger and Singh, 2011; Kindiger, 2012a). Each is characterized by a genome loss phenomenon following hybridization with tall fescue, which is then followed by a low level of parthenogenic development of an embryo in the inflorescence. The IL1 and IL2 genetic stocks exhibit few advantageous agronomic characteristics and are notable primarily for their ability to induce genome loss following hybridization. That is, there are few if any advantageous agronomic traits such as persistence, disease tolerance, drought tolerance, etc. that could be seen as favorable agronomic traits. Each IL line is free of the fungal endophyte *Epichloë* sp. or *Neotyphodium* sp. (Carroll, 1988; Moon *et al.*, 1994; Pedersen and Sleper, 1988).

Pollinations between the IL lines and tall fescue samples are described in detail elsewhere (Kindiger, 2012b, 2016) and only the general method is discussed here. IL x tall fescue pollinations are generated by hand using the IL lines as the maternal parent and applying a bulk of randomly obtained tall fescue pollen from several commercial and public cultivars in the greenhouse. Bulk tall fescue pollen from one or multiple tall fescue pollen sources is utilized to take advantage of the diversity of available tall fescue gametes across genotypes. It should be noted that the ryegrass IL lines are self-incompatible which disallows selfing and the IL lines themselves segregate for pollen sterility. To achieve efficiency in producing IL x tall fescue F1 hybrids, these two traits, found in the IL lines are of high importance. Seed generated from the hybridizations is harvested and germinated in small trays containing a BM1 all purpose potting soil mixture (Berger, CA, USA) topsoil (local product). Eighty-five liters of the BM1 mix were blended with 18.14kg of topsoil and blended by hand. Conditions for germination and plant growth were in a non-shaded, natural lighted greenhouse with a night to day temperature range from 21 C 30 C. Trays and pots with germinating seedlings and plants were watered with overhead misters for approximately 2 hours daily. The resultant F1 hybrids were eventually transferred to eight inch pots filled with the potting mix-top soil blend described above. The F1 were allowed to grow to maturity in a pollen-free environment as prior investigations has indicated that though the F1 are generally female and seed sterile; it has been observed that some haploid eggs are viable and if pollen containment conditions are not utilized, uncontrolled pollination events would confound the generation and production of DH offspring (Kindiger, 2016). The F1 hybrids in the pollen isolated greenhouse were grown to maturity and, as is typical of such hybrids, were observed to be pollen sterile; however, it is noted that some level of pollen fertility can occasionally occur (Buckner, 1960; Buckner *et al.*, 1961). If any F1 appeared to indicate some level of pollen fertility by exerting its anthers, it was removed to an adjoining greenhouse bay. When mature, the inflorescences were harvested and threshed by hand or machine to remove any stems. The cleaned seed heads were then placed in trays containing a light potting soil mix for germination and the eventual identification and selection for recovered ryegrass or tall fescue seedlings. It is important to note that embryo culture or embryo rescue methods were not employed for DH generation. Following two-to-three weeks of germination, seedlings were allowed to grow to an appropriate size to allow for phenotypic identification and eventual transplanting to pots. The germinating seedlings generally represent a mixture of ryegrass DH recoveries possessing a chromosome number of 2n=2x=14 or tall fescue DH recoveries possessing a chromosome number of 2n=6x=42. Discrimination between the ryegrass and tall fescue DH recoveries was performed by examination of phenotypic characters such as leaf width and growth habit and, as required, verified by chromosome counts. Counts were performed by methods published previously (Kindiger, 1996), then verified tall fescue DH seedlings was transferred to eight-inch pots containing a light potting soil mixture for further growth. DH tall fescue recoveries were also identified by molecular marker screenings (Kindiger, 2016).

In September 2011, 25 two-year-old F1's and one each of their respective one-year-old DH recoveries were transplanted to a non-replicated space-planted nursery at the of Kansas State University, Southeast Agricultural Research Center, Parsons, KS (Figure 1). The F1 and their respective DH offspring were transplanted adjacent to each other at a distance of 50 cm, plant center-to plant center. These closely adjacent plantings were performed

to minimize soil differences across the field that would affect the forage quality determinations. There was no intent in this experiment to evaluate forage quality across genotypes as the non-replicated nursery negated the ability to compare different genotypes in differing areas of the nursery due to anticipated field differences. Instead, the inheritance and expression of crude protein (CP), Acid digestible fiber (ADF); neutral digestible fiber (NDF) and in vitro dry matter digestibility (IVDMD); each important forage quality components, were evaluated in the F1 and their respective DH offspring. The nursery was fertilized in October with 18 kg/ha granular nitrogen and weeding was performed by hand as necessary.

Figure 1. Spaced planting orientation of the nursery in Mound Valley, KS at the Kansas State University, Southeast Agricultural Research Center. The F1 and recovered DH were transplanted on 50cm centers in order to minimize known field variability.

On April 23, 2012, 25 wet leaf samples were obtained from the F1 and their twenty-five DH offspring. At least 300 grams of fresh leaf material per individual were obtained, sealed in Ziploc® bags and posted on a next day delivery schedule to ANALab, Fulton, IL for estimations of crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF) and in vitro dry matter digestibility (IVDMD). ANALab dried the leaf materials and utilized NIR to obtain CP, ADF, NDF and IVDMD results (Table 1). A contingency chi-square test was applied using the combined F1's as the "observed" value and the combined DH values as the "expected" across all the samples to determine if the forage quality determinations of the combined F1 and DH offspring were, on average, statistically similar.

RESULTS
In this gamete selection approach, forage quality is conferred primarily by the genotype of the retained genome of the tall fescue individual that provided the single pollen grain (gamete) that generated the IL x tall fescue hybrid. Forage quality components were evaluated at a single location, in an un-replicated, spaced planting nursery. Another approach to determine the expression of the forage quality traits could be performed with vegetative propagation of the F1 plants and the DH offspring, across several locations. This would allow an evaluation of forage quality expression across multiple selection pressures and how the genotypes are reacting to those differences.

Table 1. Percent crude protein, ADF, NDF and IVDMD of F1 (LF) hybrids and their respective recovered DH lines.

Pair Set	Sample	CP[1]	ADF[2]	NDF[3]	IVDMD[4]
1	LF84	20.97	27.69	48.93	72.75
	DH117B	15.77	32.18	56.07	64.25
2	LF74	19.74	29.9	53.52	68.04
	DH105	19.62	29.23	50.08	70.77
3	LF36	23.27	27.09	47.75	72.37
	DH118B	25.18	32.39	55.16	65.14
4	LF89	19.32	26.67	56.65	73.10
	DH133B	19.84	29.47	55.83	67.56
5	LF102	19.73	27.57	49.65	72.55
	DH110B	19.53	31.17	54.89	75.91
6	LF90	20.24	27.59	50.72	71.89
	DH120B	20.68	28.21	48.45	66.82
7	LH39	21.28	27.92	49.98	68.63
	DH113B	19.89	30.76	52.84	66.52
8	LF117	17.45	31.25	56.59	67.18
	DH112B	19.06	31.18	52.98	66.56
9	LF99	18.86	28.91	52.41	69.10
	DH108	20.68	29.51	50.29	69.74
10	LF65	15.88	31.72	55.39	65.96
	DH119	17.18	31.91	54.15	62.07
11	LF116	17.41	30.48	54.86	65.04
	DH115B	17.48	31.38	53.43	68.51
12	LF51	17.33	31.63	55.28	64.92
	DH129B	15.64	29.21	48.09	67.96
13	LF94	16.12	30.8	53.92	64.81
	DH124B	13.89	32.24	55.88	59.70
14	LF118	18.69	28.74	52.21	67.24
	DH126B	17.11	26.63	47.44	66.71
15	LF107	16.71	30.92	53.37	64.55
	DH131B	18.63	30.68	57.02	68.36
16	LF38	18.35	31.6	54.01	62.84
	DH132B	17.09	32.13	54.34	63.27
17	LF100	14.74	30.59	53.49	61.98
	DH138B	17.85	28.72	48.94	68.84
18	LF49	19.28	31.01	53.88	63.16
	DH130B	19.75	29.81	52.12	64.47
19	LF72	19.31	29.79	52.62	66.88
	DH147B	18.39	29.11	49.68	63.61
20	LF78	18.75	31.24	52.96	64.28
	DH144B	17.42	32.23	58.03	67.09
21	LF93	18.27	29.79	52.99	69.85
	DH142B	20.8	29.71	53.66	67.41
22	LF76	18.35	30.19	50.59	68.18
	DH154B	18.45	29.46	51.04	65.79
23	LF109	16.92	32.54	56.44	64.40
	DH153B	18.88	30.37	51.53	66.40
24	LF80	14.67	29.9	50.74	63.65
	DH152B	16.29	28.64	49.01	66.11
25	LF111	15.83	31.54	55.81	61.49
	DH150B	15.47	30.32	54.35	61.83

[1]CP = percent crude protein

[2]ADF = percent acid digestible fiber

[3]NDF = percent neutral digestible fiber

[4]IVDMD – percent in vitro dry matter digestibility

Chi-square statistics were applied to the ANA Labs forage analysis results of CP, ADF, NDF and IVDMD. Chi-square values were 0.99 (p< 0.05) for all traits, indicating no significant difference between the parental F1 and its respective DH offspring. This indicates that the inheritance and expression of the forage quality traits was primarily due to the tall fescue genome and was not compromised by the loss of the IL ryegrass genome. In other words, the retention of the tall fescue genome and all its forage quality traits, has full transfer to the dihaploid tall fescue genotype and expression of those traits is not hugely affected by the loss of any genes affecting any forage quality traits that are conferred by the presence/loss of the ryegrass genome. As an example, the estimated CP component of F1 hybrid LH74 was 19.74% while that of its DH recovery was 19.62%. The values for ADF, NDF and IVDMD for the F1, LH74 were 29.9, 53.52 and 68.04%, respectively, while those of its DH recovery were 29.35, 53.74 and 65.99%, respectively. The loss of the ryegrass genome, with few if any favorable forage attributes, did not dramatically alter the forage quality of the recovered tall fescue DH when the ryegrass genome was lost. Most of the forage qualities were retained by the retention and doubling of the tall fescue genome in the dihaploid state. This represents the power of the 'gamete' selection approach and the ultimate development of dihaploid tall fescue that fixes the genotype of that tall fescue gamete in a homozygous, dihaploid state. The other F1 utilized in this study exhibited similar results (Table 1). These data suggest little difference between the forage quality characteristics of an F1 and its DH offspring. The lack of significant difference between the F1 and the respective DH line also indicates the IL line provided little forage quality attributes to the F1 parent. This outcome is advantageous as the exhibited forage attributes of each F1 and DH recovery can be attributed primarily by the genotype of the tall fescue gamete. It is also important to note that these data also suggest that selection for these forage qualities can be performed directly on the F1 and not delayed until the development of a DH recovery.

Presuming that F1 possessing higher CP are more desirable than those with lower CP, that higher ADF is better than lower ADF, that lower NDF is superior to higher NDF, and that higher IVDMD values are superior to lower IVDMD values, then the F1 lines LF84, LF36, LF102 and LF89 of this study are the superior candidates for future DH recovery. It appears that evaluating quality traits against the F1 and identifying the superior F1 individuals in the first year of a study would greatly reduce the number of F1 necessary for DH generation and prioritize the research on F1 possessing superior genotypes. Once the F1 with the superior genotypes are identified, future efforts can be focused on obtaining DH lines from superior F1. This would also reduce the need for nursery space and for the extra year of effort that would have been focused on the maintenance of F1 and the development of DH lines exhibiting lesser quality attributes. Following this approach, the identified F1 would produce DH lines DH122B, DH118B; DH110B and DH133B that could be used for future research, hybridizations and breeding investigations.

These results suggest that a gamete selection approach, with the selection at the F1 level, will be highly effective and provide a good estimation of the CP, ADF, NDF and IVDMD qualities of any DH line that is derived from any particular F1 hybrid. As the DH recoveries retain most of the CP, ADH, NDH and IVDMD levels identified in their F1 parents, the most efficient utilization of this method would be to initiate selection of these traits on the F1 individuals rather than postponing selection at the DH recovery stage. By identifying F1 with elevated forage quality traits, the number and process of generating DH exhibiting lesser forage quality traits can be reduced or eliminated as only the F1 with the superior forage quality levels would be moved forward into a DH generation portion of the selection program.

DISCUSSION

Gamete selection greatly simplifies multi-gene or quantitative trait transfer within a breeding program when compared to traditional backcross, selfing or recurrent selection breeding methods (Maluszynski *et al.*, 2003). As useful as DH breeding approaches can be (Brummer and Casler, 2009; Amini *et al.*, 2011), these approaches are limited by the unavailability of such a methodology in many species; and as such, the generation of haploid or dihaploid lines through a gamete selection approach represents an unutilized approach for plant breeding and genetic analysis research (Dunwell, 2010). The proposed gamete selection breeding approach applied in this research offers a novel method for improving tall fescue germplasm. Differences in the forage values of CP, ADF, NDF and IVDMD across 25 F1 and 25 of their respective recovered DH lines is not significantly different. This suggests that selection for

forage quality traits will be more efficient when performed at the F1 stage than at the subsequent DH generation stage. Since DH recoveries are a low frequency event, the effort expended on DH generation will be more efficient if F1 expressing superior forage quality traits were selected for future DH generation. F1 with a superior forage quality genotype will produce, homozygous DH lines possessing superior forage quality genotypes.

Though IL1 and IL2 can be utilized to generate DH lines, the frequency of generation is low, likely less than 1% (Kindiger and Singh, 2011), the ability to generate large numbers of F1 and the focus on the identification and selection of only superior F1 make this approach a highly beneficial breeding system. The generated IL x TF F1 hybrids can number in the thousands from a single IL x tall fescue hybridization. Due to the perennial nature of the F1, vegetative propagation can allow multi-location testing of identical genotypes, and numerous inflorescences can be available year after year. These advantages counter the low incidence of DH line recoveries from an F1. It should also be noted that the generation of a large number of DH from an F1 is unnecessary as most DH generated from a particular F1 will likely be identical in their genotype, barring mitotic inter-genomic recombination events. The successful generation of only one DH per F1 is all that is necessary to fix and propagate the genotype of a superior F1 hybrid. Gamete selection of forage quality traits in the F1 and the comparison of that same trait in the DH recoveries suggest this approach will be viable for the improvement of forage quality traits in tall fescue. In addition, the generation and availability of a DH line possessing a fixed genotype for that trait would prevent the loss or segregation of those genes if selfing within the DH line were utilized for seed increase. As with a hybrid maize production system, it may also be possible to combine these tall fescue DH lines for the production of tall fescue synthetics or true F1 hybrids that will capitalize on these and additional genotypic heterotic effects (Pinnell *et al.*, 1952; Birchler *et al.*, 2010). The replicated performance of this selection process over years and locations will provide additional forage quality information to the breeder.

The materials and procedures described herein apply directly to the breeding and selection of improved tall fescue genotypes. The sampling of hundreds of thousands of gametes via the pollen grains, each segregating for a myriad of genotypes from a single tall fescue individual or population along with selection on a sporophyte basis, represents a low input, low cost, rapid selection strategy that can be implemented across a diversity of environments. It is anticipated that, when applied correctly, this approach will not only be effective for the selection of forage quality attributes but will also be effective for the selection of both qualitative and quantitative traits. These might include maturity, drought tolerance, disease resistance, grazing persistence and forage yield. Such complex, quantitative traits could immediately be transferred to their DH recoveries.

The *Festuca* genus, the largest within the Loliinae subtribe of the Poaceae family, contains more than 500 species of temperate grasses (Inda *et al.*, 2008). The genus varies substantially in ploidy levels, ranging from diploid (2n = 2x = 14) to dodecaploid (2n = 12x = 84), with the vast majority of species being allopolyploid. It is anticipated that gamete selection within the F1 generated by many of these species will also be highly effective. Studies are now underway to evaluate this hypothesis.

The following patents have been assigned and awarded to the USDA-ARS for the technology presented in this publication: US Patent No. 20,100,083,400; 9.958,107; New Zealand Patent No. 592035; Australian Patent No. 2015246971; Canadian Patent No. 2,738,762. Recent investigations utilizing the inducer dihaploid selection approach in meadow fescue and festulolium have been promising and suggest this approach will be equally effective for selection and improvement of forage quality attributes in these species. U.S patent application SN 16/575,792 has been submitted to the USPVPO and foreign patent submissions are pending.

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